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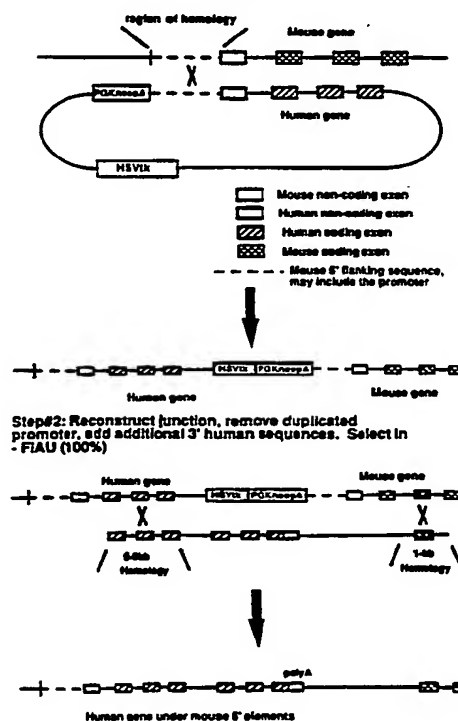
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(54) Title: METHOD FOR HOMOLOGOUS RECOMBINATION IN ANIMAL AND PLANT CELLS

* Step#1, homologous recombination: Adding the human replacement with an insertion vector.



(57) Abstract

A method for producing animal cells which contain a desired gene sequence which has been inserted into a predetermined gene sequence by homologous recombination. The method permits the production of animal cells which have subtle and precise modifications of gene sequence and expression.

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7 **TITLE OF THE INVENTION:**
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11 Method for Homologous Recombination in Animal
12 and Plant Cells
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15 **CROSS-REFERENCE TO RELATED APPLICATIONS:**
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18 This application is a continuation-in-part application
19 of U.S. Patent Application Serial No. 07/537,458, filed on
20 June 14, 1990.
21

22 **FIELD OF THE INVENTION:**
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24 The invention is directed toward recombinant DNA
25 technology, and more specifically, toward methods for
26 modifying endogenous genes in a chimeric or transgenic
27 animal or plant. The invention further pertains to the
28 animals/plants produced through application of the method,
29 and to the use of the method in medicine and agriculture.
30 This invention was supported by Government funds. The
31 Government has certain rights in this invention.
32

33 **BACKGROUND OF THE INVENTION:**
34

35 **I. Chimeric and Transgenic Animals**
36

37 Recent advances in recombinant DNA and genetic
38 technologies have made it possible to introduce and express
39 a desired gene sequence in a recipient animal. Through the
40 use of such methods, animals have been engineered to contain

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1 gene sequences that are not normally or naturally present in
2 an unaltered animal. The techniques have also been used to
3 produce animals which exhibit altered expression of
4 naturally present gene sequences.

5 The animals produced through the use of these methods
6 are known as either "chimeric" or "transgenic" animals. In
7 a "chimeric" animal, only some of the animal's cells contain
8 and express the introduced gene sequence, whereas other
9 cells have been unaltered. The capacity of a chimeric
10 animal to transmit the introduced gene sequence to its
11 progeny depends upon whether the introduced gene sequences
12 are present in the germ cells of the animal. Thus, only
13 certain chimeric animals can pass along the desired gene
14 sequence to their progeny.

15 In contrast, all of the cells of a "transgenic" animal
16 contain the introduced gene sequence. Consequently, a
17 transgenic animal is capable of transmitting the introduced
18 gene sequence to its progeny.

19
20 **II. Production of Transgenic Animals:**
21 **Microinjection Methods**
22

23 The most widely used method through which transgenic
24 animals have been produced involves injecting a DNA molecule
25 into the male pronucleus of a fertilized egg (Brinster, R.L.
26 et al., Cell 27:223 (1981); Costantini, F. et al., Nature
27 294:92 (1981); Harbers, K. et al., Nature 293:540 (1981);
28 Wagner, E.F. et al., Proc. Natl. Acad. Sci. (U.S.A.) 78:5016
29 (1981); Gordon, J.W. et al., Proc. Natl. Acad. Sci. (U.S.A.)
30 73:1260 (1976)).

31 The gene sequence being introduced need not be incor-
32 porated into any kind of self-replicating plasmid or virus
33 (Jaenisch, R., Science, 240:1468-1474 (1988)). Indeed, the

1 presence of vector DNA has been found, in many cases, to be
2 undesirable (Hammer, R.E. et al., Science 235:53 (1987);
3 Chada, K. et al., Nature 319:685 (1986); Kollias, G. et al.,
4 Cell 46:89 (1986); Shani, M., Molec. Cell. Biol. 6:2624
5 (1986); Chada, K. et al., Nature 314:377 (1985); Townes, T.
6 et al., EMBO J. 4:1715 (1985)).

7 After being injected into the recipient fertilized egg,
8 the DNA molecules are believed to recombine with one another
9 to form extended head-to-tail concatemers. It has been
10 proposed that such concatemers occur at sites of double-
11 stranded DNA breaks at random sites in the egg's
12 chromosomes, and that the concatemers are inserted and
13 integrated into such sites (Brinster, R.L. et al., Proc.
14 Natl. Acad. Sci. (U.S.A.) 82:4438 (1985)). Although it is,
15 thus, possible for the injected DNA molecules to be
16 incorporated at several sites within the chromosomes of the
17 fertilized egg, in most instances, only a single site of
18 insertion is observed (Jaenisch, R., Science, 240:1468-1474
19 (1988); Meade, H. et al. (U.S. Patent 4,873,316)).

20 Once the DNA molecule has been injected into the
21 fertilized egg cell, the cell is implanted into the uterus
22 of a recipient female, and allowed to develop into an
23 animal. Since all of the animal's cells are derived from
24 the implanted fertilized egg, all of the cells of the
25 resulting animal (including the germ line cells) shall
26 contain the introduced gene sequence. If, as occurs in
27 about 30% of events, the first cellular division occurs
28 before the introduced gene sequence has integrated into the
29 cell's genome, the resulting animal will be a chimeric
30 animal.

31 By breeding and inbreeding such animals, it has been
32 possible to produce heterozygous and homozygous transgenic
33 animals. Despite any unpredictability in the formation of

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1 such transgenic animals, the animals have generally been
2 found to be stable, and to be capable of producing offspring
3 which retain and express the introduced gene sequence.

4 Since microinjection causes the injected DNA to be
5 incorporated into the genome of the fertilized egg through
6 a process involving the disruption and alteration of the
7 nucleotide sequence in the chromosome of the egg at the
8 insertion site, it has been observed to result in the
9 alteration, disruption, or loss of function of the
10 endogenous egg gene in which the injected DNA is inserted.
11 Moreover, substantial alterations (deletions, duplications,
12 rearrangements, and translocations) of the endogenous egg
13 sequences flanking the inserted DNA have been observed
14 (Mahon, K.A. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:1165
15 (1988); Covarrubias, Y. et al., Proc. Natl. Acad. Sci.
16 (U.S.A.) 83:6020 (1986); Mark, W. et al., Cold Spr. Harb.
17 Symp. Quant. Biol. 50:453 (1985)). Indeed, lethal mutations
18 or gross morphological abnormalities have been observed
19 (Jaenisch, R., Science 240:1468-1474 (1988); First, N.L. et
20 al., Amer. Meat Sci. Assn. 39th Reciprocal Meat Conf. 39:41
21 (1986)).

22 Significantly, it has been observed that even if the
23 desired gene sequence of the microinjected DNA molecule is
24 one that is naturally found in the recipient egg's genome,
25 integration of the desired gene sequence rarely occurs at
26 the site of the natural gene (Brinster, R.L. et al., Proc.
27 Natl. Acad. Sci. (U.S.A.) 86:7087-7091 (1989)). Moreover,
28 introduction of the desired gene sequence does not generally
29 alter the sequence of the originally present egg gene.

30 Although the site in the fertilized egg's genome into
31 which the injected DNA ultimately integrates cannot be
32 predetermined, it is possible to control the expression of
33 the desired gene sequence such that, in the animal,

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expression of the sequence will occur in an organ or tissue specific manner (reviewed by Westphal, H., FASEB J. 3:117 (1989); Jaenisch, R., Science 240:1468-1474 (1988)).

The success rate for producing transgenic animals is greatest in mice. Approximately 25% of fertilized mouse eggs into which DNA has been injected, and which have been implanted in a female, will become transgenic mice. A lower rate has been thus far achieved with rabbits, sheep, cattle, and pigs (Jaenisch, R., Science 240:1468-1474 (1988); Hammer, R.E. et al., J. Animal. Sci. 63:269 (1986); Hammer, R.E. et al., Nature 315:680 (1985); Wagner, T.E. et al., Theriogenology 21:29 (1984)). The lower rate may reflect greater familiarity with the mouse as a genetic system, or may reflect the difficulty of visualizing the male pronucleus of the fertilized eggs of many farm animals (Wagner, T.E. et al., Theriogenology 21:29 (1984)).

Thus, the production of transgenic animals by microinjection of DNA suffers from at least two major drawbacks. First, it can be accomplished only during the single-cell stage of an animal's life. Second, it requires the disruption of the natural sequence of the DNA, and thus is often mutagenic or teratogenic (Gridley, T. et al., Trends Genet. 3:162 (1987)).

III. Production of Chimeric and Transgenic Animals: Recombinant Viral and Retroviral Methods

Chimeric and transgenic animals may also be produced using recombinant viral or retroviral techniques in which the gene sequence is introduced into an animal at a multi-cell stage. In such methods, the desired gene sequence is introduced into a virus or retrovirus. Cells which are infected with the virus acquire the introduced gene

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1 sequence. If the virus or retrovirus infects every cell of
2 the animal, then the method results in the production of a
3 transgenic animal. If, however, the virus infects only some
4 of the animal's cells, then a chimeric animal is produced.

5 The general advantage of viral or retroviral methods of
6 producing transgenic animals over those methods which
7 involve the microinjection of non-replicating DNA, is that
8 it is not necessary to perform the genetic manipulations at
9 a single cell stage. Moreover, infection is a highly
10 efficient means for introducing the DNA into a desired cell.

11 Recombinant retroviral methods for producing chimeric or
12 transgenic animals have the advantage that retroviruses
13 integrate into a host's genome in a precise manner,
14 resulting generally in the presence of only a single
15 integrated retrovirus (although multiple insertions may
16 occur). Rearrangements of the host chromosome at the site
17 of integration are, in general, limited to minor deletions
18 (Jaenisch, R., Science 240:1468-1474 (1988); see also,
19 Varmus, H., In: RNA Tumor Viruses (Weiss, R. et al., Eds.),
20 Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 369-
21 512 (1982)). The method is, however, as mutagenic as micro-
22 injection methods.

23 Chimeric animals have, for example, been produced by
24 incorporating a desired gene sequence into a virus (such as
25 bovine papilloma virus or polyoma) which is capable of
26 infecting the cells of a host animal. Upon infection, the
27 virus can be maintained in an infected cell as an
28 extrachromosomal episome (Elbrecht, A. et al., Molec. Cell.
29 Biol. 7:1276 (1987); Lacey, M. et al., Nature 322:609
30 (1986); Leopold, P. et al., Cell 51:885 (1987)). Although
31 this method decreases the mutagenic nature of
32 chimeric/transgenic animal formation, it does so by
33 decreasing germ line stability, and increasing oncogenicity.

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1 Pluripotent embryonic stem cells (referred to as "ES"
2 cells) are cells which may be obtained from embryos until
3 the early post-implantation stage of embryogenesis. The
4 cells may be propagated in culture, and are able to
5 differentiate either in vitro or in vivo upon implantation
6 into a mouse as a tumor. ES cells have a normal karyotype
7 (Evans, M.J. et al., Nature 292:154-156 (1981); Martin, G.R.
8 et al., Proc. Natl. Acad. Sci. (U.S.A.) 78:7634-7638
9 (1981)).

10 Upon injection into a blastocyst of a developing embryo,
11 ES cells will proliferate and differentiate, thus resulting
12 in the production of a chimeric animal. ES cells are
13 capable of colonizing both the somatic and germ-line
14 lineages of such a chimeric animal (Robertson, E. et al.,
15 Cold Spring Harb. Conf. Cell Prolif. 10:647-663 (1983);
16 Bradley A. et al., Nature 309:255-256 (1984); Bradley, A. et
17 al., Curr. Top. Devel. Biol. 20:357-371 (1986); Wagner, E.F.
18 et al., Cold Spring Harb. Symp. Quant. Biol. 50:691-700
19 (1985); (all of which references are incorporated herein by
20 reference).

21 In this method, ES cells are cultured in vitro, and
22 infected with a viral or retroviral vector containing the
23 gene sequence of interest. Chimeric animals generated with
24 retroviral vectors have been found to have germ cells which
25 either lack the introduced gene sequence, or contain the
26 introduced sequence but lack the capacity to produce progeny
27 cells capable of expressing the introduced sequence (Evans,
28 M.J. et al., Cold Spring Harb. Symp. Quant. Biol. 50:685-689
29 (1985); Stewart, C.L. et al., EMBO J. 4:3701-3709 (1985);
30 Robertson, L. et al., Nature (1986); which references are
31 incorporated herein by reference).

32 Because ES cells may be propagated in vitro, it is
33 possible to manipulate such cells using the techniques of

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1 somatic cell genetics. Thus, it is possible to select ES
2 cells which carry mutations (such as in the hprt gene
3 (encoding hypoxanthine phosphoribosyl transferase) (Hooper,
4 M. et al., Nature 326:292-295 (1987); Kuehn, M.R. et al.,
5 Nature 326:295-298 (1987)). Such selected cells can then be
6 used to produce chimeric or transgenic mice which fail to
7 express an active HPRT enzyme, and thus provide animal
8 models for diseases (such as the Lesch-Nyhan syndrome which
9 is characterized by an HPRT deficiency) (Doetschman, T. et
10 al., Proc. Natl. Acad. Sci. (U.S.A.) 85:8583-8587 (1988)).

11 As indicated above, it is possible to generate a
12 transgenic animal from a chimeric animal (whose germ line
13 cells contain the introduced gene sequence) by inbreeding.

14 The above-described methods permit one to screen for th
15 desired genetic alteration prior to introducing the trans-
16 fected ES cells into the blastocyst. One drawback of these
17 methods, however, is the inability to control the site or
18 nature of the integration of the vector.

20 IV. Production of Chimeric and Transgenic Animals: 21 Plasmid Methods

22
23 The inherent drawbacks of the above-described methods
24 for producing chimeric and transgenic animals have caused
25 researchers to attempt to identify additional methods
26 through which such animals could be produced.

27 Gossler, A. et al., for example, have described the use
28 of a plasmid vector which had been modified to contain the
29 gene for neomycin phosphotransferase (nptII gene) to
30 transfect ES cells in culture. The presence of the nptII
31 gene conferred resistance to the antibiotic G418 to ES cells
32 that had been infected by the plasmid (Gossler, A. et al.,
33 Proc. Natl. Acad. Sci. (U.S.A.) 83:9065-9069 (1986), which

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reference is incorporated herein by reference). The chimeric animals which received the plasmid and which became resistant to G418, were found to have integrated the vector into their chromosomes. Takahashi, Y. et al. have described the use of a plasmid to produce chimeric mice cells which expressed an avian crystallin gene (Development 102:258-269 (1988), incorporated herein by reference). The avian gene was incorporated into a plasmid which contained the nptII gene. Resulting chimeric animals were found to express the avian gene.

V. Introduction of Gene Sequences into Somatic Cells

DNA has been introduced into somatic cells to produce variant cell lines. hprt-deficient Chinese hamster ovary (CHO) cells have been transformed with the CHO hprt gene in order to produce a prototrophic cell line (Graf, L.H. et al., Somat. Cell Genet. 5:1031-1044 (1979)). Folger et al. examined the fate of a thymidine kinase gene (tk gene) which had been microinjected into the nuclei of cultured mammalian cells. Recipient cells were found to contain from 1 to 100 copies of the introduced gene sequence integrated as concatemers at one or a few sites in the cellular genome (Folger, K.R. et al., Molec. Cell. Biol. 2:1372-1387 (1982)). DNA-mediated transformation of an RNA polymerase II gene into Syrian hamster cells has also been reported (Ingles, C. et al., Molec. Cell. Biol. 2:666-673 (1982)).

Plasmids conferring host neomycin resistance and guanosine phosphotransferase activity have been transfected into Chinese hamster ovary cells to generate novel cell lines (Robson, C.N. et al., Mutat. Res. 163:201-208 (1986)).

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VI. Chimeric or Transgenic Plants

Extensive progress has been made in recent years in the fields of plant cell genetics and gene technology. For many genera of plants, protoplast regeneration techniques can be used to regenerate a plant from a single cell (Friedt, W. et al. Prog. Botany 49:192-215 (1987); Brunold, C. et al., Molec. Gen. Genet. 208:469-473 (1987); Durand, J. et al., Plant Sci. 62:263-272 (1989) which references are incorporated herein by reference).

Several methods can be used to deliver and express a foreign gene into a plant cell. The most widely used method employs cloning the desired gene sequence into the Ti plasmid of the soil bacterium A. tumorigenicus (Komari, T. et al., J. Bacteriol. 166:88-94 (1986); Czako, M. et al., Plant Mol. Biol. 6:101-109 (1986); Jones, J.D.G. et al., EMBO J. 4:2411-2418 (1985); Shahin, E.A. et al., Theor. Appl. Genet. 73:164-169 (1986)). The frequency of transformation may be as high as 70%, depending upon the type of plant used (Friedt, W. et al. Prog. Botany 49:192-215 (1987)).

Plant viruses have also been exploited as vectors for the delivery and expression of foreign genes in plants. The cauliflower mosaic virus (Brisson, N. et al., Nature 310:511-514 (1984) has been particularly useful for this purpose (Shah, D.M. et al., Science 233:478-481 (1986); Shewmaker, C.K. et al., Virology 140:281-288 (1985). Vectors have also been prepared from derivatives of RNA viruses (French, R. et al., Science 231:1294-1297 (1986)).

Techniques of microinjection (Crossway, A. et al., Molec. Gen. Genet. 202:179-185 (1986); Potrykus, I. et al., Molec. Gen. Genet. 199:169-177 (1985)), have been used to accomplish the direct transfer of gene sequences into plant

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1 cells. Transformation with a plasmid capable of site
2 specific recombination has been used to introduce gene
3 sequences into Aspergillus (May, G.S., J. Cell Biol.
4 109:2267-2274 (1989); which reference is incorporated herein
5 by reference).

6 Electroporation has been identified as a method for
7 introducing DNA into plant cells (Fromm, M.E., et al., Proc.
8 Natl. Acad. Sci. (U.S.A.) 82:5824-5828 (1985); Fromm, M.E.
9 et al., Nature 319:791-793 (1986); Morikawa, H. et al., Gene
10 41:121-124 (1986); Langridge, W.H.R. et al., Theor. Appl.
11 Genet. 67:443-455 (1984)).

12 Gross genetic mutations can be produced in plant cells
13 using transposable elements (Saedler, H. et al., EMBO J.
14 4:585-590 (1985); Peterson, P.A., BioEssays 3:199-204
15 (1985)). Such elements can initiate chromosomal
16 rearrangements, insertions, duplications, deletions, etc.
17 Chimeric plants can be regenerated from such cells using the
18 procedures described above.

19 A major deficiency of present methods for gene
20 manipulation in plants is the difficulty of selecting the
21 desired recombinant cell (Brunold, C. et al., Molec. Gen.
22 Genet. 208:469-473 (1987)). In an attempt to address this
23 deficiency, kanamycin resistance and nitrate reductase
24 deficiency have been used as selectable markers (Brunold, C.
25 et al., Molec. Gen. Genet. 208:469-473 (1987)).
26
27

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VII. Conclusions

The application of the above-described technologies has the potential to produce types of plants and animals which cannot be produced through classical genetics. For example, animals can be produced which suffer from human diseases (such as AIDS, diabetes, cancer, etc.), and may be valuable in elucidating therapies for such diseases. Chimeric and transgenic plants and animals have substantial use as probes of natural gene expression. When applied to livestock and food crops, the technologies have the potential of yielding improved food, fiber, etc.

Despite the successes of the above-described techniques, a method for producing chimeric or transgenic plants and animals which was less mutagenic, and which would permit defined, specific, and delicate manipulation of the inserted gene sequence at a specific chromosomal location would be highly desirable.

BRIEF DESCRIPTION OF THE FIGURES:

Figure 1 illustrates the use of replacement vectors and insertion vectors in gene targeting. Figure 1A is a diagrammatical representation of the use of a replacement vector in gene targeting; Figure 1B illustrates the use of an insertion vector to produce subtle mutations in a desired gene sequence.

Figure 2 is a diagrammatical representation of a DNA molecule which has a region of heterology located at a proposed insertion site. Figure 2A shows a construct with a 2 kb region of heterology. Figure 2B shows a construct with a 26 base long region of heterology which has been

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1 linearized at the center of the region of heterology.
2 Figure 2C shows a construct with a region of heterology
3 located internal to the region of homology at which
4 recombination is desired. In Figure 2C, the normal BamHI
5 site of the vector has been changed to an NheI site and the
6 normal EcoRI site of the vector has been changed to a BamHI
7 site. The vector is linearized with XhoI.

8 Figure 3 is a diagrammatical representation of the
9 mechanism through which a "humanized" gene may be introduced
10 into a chromosomal gene sequence in a one step method.

11 Figure 4 is a diagrammatical representation of the
12 mechanism through which a large gene may be introduced into
13 a chromosomal gene sequence so as to place the gene under
14 the transcriptional control of a heterologous promoter (for
15 example, to place a human gene under the control of a mouse
16 gene). The first step is additive and the second is a
17 replacement event. Figure 4A shows the first step of the
18 process; Figure 4B shows the second step of the process.
19 The repair recombination event may be configured to remove
20 all of the mouse coding exons if desired.

21 Figure 5 is a diagrammatical representation of the use of
22 a positive selection/ negative selection "cassette" to
23 introduce subtle mutations into a chromosome.

24 Figure 6 is a diagrammatical representation of a multi-
25 step method (Figures 6A-6E) for introducing small or large
26 desired gene sequences into a contiguous region of a cell's
27 genome. The figure illustrates a vector capable of
28 facilitating the sequential addition of overlapping clones
29 to construct a large locus. Every step is selectable.
30 Subsequent additions may be made by returning to steps 4 and
31 5 as many times as required, selecting for insertion in HAT
32 medium, and for repair in media supplemented with 6

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1 thioguanine. This procedure may also be accomplished at the
2 other end of the locus if required.

3 Figure 7 is a diagrammatical representation of the
4 vectors used in a co-electroporation experiment to mutate
5 the hprt gene.

6 Figure 8 illustrates the predicted structure of the hprt
7 gene following homologous recombination of the IV6.8 vector.
8 HR is the predicted size fragment indicative of the
9 homologous recombination event. End, D is the endogenous
10 fragment, duplicated by the recombination event. End is the
11 predicted flanking fragment detected by the partial cDNA
12 probe used in these experiments.

13 Figure 9 shows the reversion of homologous recombinants
14 generated with insertion vectors.

15 Figure 10 illustrates the use of Poly A selection as a
16 means for selecting homologous recombination events.

17 Figure 11 illustrates the use of the invention to
18 introduce insertions into the sequence of a desired gene of
19 a cell. Figure 11A is a diagram of the c-src locus showing
20 relevant restriction sites (E=EcoRI; N=NcoI; X=XhoI;
21 H=HindIII; B=BamHI; Nh=NheI). Figure 11B illustrates the
22 src 14 vector used to introduce mutations into the c-src
23 locus; Figure 11C illustrates the subtle mutation introduced
24 through the use of this vector.

25 Figure 12 illustrates the use of the invention to
26 introduce substitutions into the sequence of a desired gene
27 of a cell. Figure 12A is a diagram of the c-src locus
28 showing relevant restriction sites (E=EcoRI; N=NcoI; X=XhoI;
29 H=HindIII; B=BamHI; Nh=NheI). Figure 12B illustrates the
30 src 33 vector used to introduce mutations into the c-src
31 locus; Figure 12C illustrates the subtle mutation introduced
32 through the use of this vector.

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Figure 13 illustrates a comparison between targeted and random recombinational events. In a random recombinational event, although concatemers can excise duplications, one copy of the vector must remain in the genome. In contrast, in a targeted recombinational event, all sequences, except the desired sequence is excised from the genome.

SUMMARY OF THE INVENTION:

The present invention provides a method for obtaining a desired animal or non-fungal plant cell which contains a predefined, specific and desired alteration in its genome. The invention further pertains to the non-human animals and plants which may be produced from such cells. The invention additionally pertains to the use of such non-human animals and plants, and their progeny in research, medicine, and agriculture.

In detail, the invention provides a method for obtaining a desired animal or non-fungal plant cell which contains a desired non-selectable gene sequence inserted within a predetermined gene sequence of the cell's genome, which method comprises:

A. incubating a precursor cell with a DNA molecule containing the desired non-selectable gene sequence, wherein the DNA molecule additionally contains two regions of homology which flank the desired gene sequence, and which are sufficient to permit the desired gene sequence to undergo homologous recombination with the predetermined gene sequence of the genome of the precursor cell;

B. causing the DNA molecule to be introduced into the precursor cell;

C. permitting the introduced DNA molecule to undergo homologous recombination with the predetermined gene

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1 sequence of the genome of the precursor cell to thereby
2 produce the desired cell wherein the desired non-selectable
3 gene sequence has been inserted into the predetermined gene
4 sequence; and

5 D. recovering the desired cell.

6 The invention further includes the embodiments of the
7 above-described method wherein the DNA molecule contains a
8 detectable marker gene sequence, and/or wherein the DNA
9 molecule is introduced into the precursor cell by subjecting
10 the precursor cell and the DNA molecule to electroporation
11 (especially wherein in step B, the precursor cell is
12 simultaneously subjected to electroporation with a second
13 DNA molecule, the second DNA molecule containing a
14 detectable marker gene sequence).

15 The invention further includes the embodiments of the
16 above-described method wherein the desired cell is a non-
17 fungal plant cell, a somatic animal cell (especially one
18 selected from the group consisting of a chicken, a mouse, a
19 rat, a hamster, a rabbit, a sheep, a goat, a fish, a pig, a
20 cow or bull, a non-human primate and a human), a pluripotent
21 animal cell (especially one selected from the group
22 consisting of a chicken, a mouse, a rat, a hamster, a
23 rabbit, a sheep, a goat, a fish, a pig, a cow or bull, and
24 a non-human primate). The invention includes with the
25 embodiment wherein the pluripotent cell is an embryonic stem
26 cell.

27 The invention also includes the embodiments of the
28 above-described methods wherein the desired gene sequence is
29 substantially homologous to the predetermined gene sequence
30 of the precursor cell and/or wherein the desired gene
31 sequence is an analog (and especially a human analog) of the
32 predetermined sequence of the precursor cell.

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1 The invention also includes the embodiment wherein the
2 desired gene sequence encodes a protein selected from the
3 group consisting of: a hormone, an immunoglobulin, a
4 receptor molecule, a ligand of a receptor molecule, and an
5 enzyme.

6 The invention also includes a non-fungal plant cell
7 which contains an introduced recombinant DNA molecule
8 containing a desired gene sequence, the desired gene
9 sequence being flanked by regions of homology which are
10 sufficient to permit the desired gene sequence to undergo
11 homologous recombination with a predetermined gene sequence
12 of the genome of the cell.

13 The invention also includes a non-human animal cell
14 which contains an introduced recombinant DNA molecule
15 containing a desired gene sequence, the desired gene
16 sequence being flanked by regions of homology which are
17 sufficient to permit the desired gene sequence to undergo
18 homologous recombination with a predetermined gene sequence
19 of the genome of the cell.

20 The invention also includes the desired cell produced by
21 any of the above-described methods.

22 The invention also includes a non-human animal
23 containing a cell derived from the above-described desired
24 cell, or a descendant thereof, wherein the animal is either
25 a chimeric or a transgenic animal, and particularly includ s
26 the embodiment wherein the non-human animal and the desired
27 cell are of the same species, and wherein the species is
28 selected from the group consisting of: a chicken, a mouse,
29 a rat, a hamster, a rabbit, a sheep, a goat, a fish, a pig,
30 a cow or bull, and a non-human primate.

31 The invention also includes a non-fungal plant
32 containing a cell derived from the above-described desired

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1 non-fungal plant cell, wherein said non-fungal plant is
2 either a chimeric or a transgenic plant.

3 The invention also includes a method of gene therapy
4 which comprises introducing to a recipient in need of such
5 therapy, a desired non-selectable gene sequence, the method
6 comprising:

7 A. providing to the recipient an effective amount
8 of a DNA molecule containing the desired non-selectable gene
9 sequence, wherein the DNA molecule additionally contains two
10 regions of homology which flank the desired gene sequence,
11 and which are sufficient to permit the desired gene sequence
12 to undergo homologous recombination with a predetermined
13 gene sequence present in a precursor cell of the recipient;

14 B. permitting the DNA molecule to be introduced
15 into the precursor cell;

16 C. permitting the introduced DNA molecule to
17 undergo homologous recombination with the predetermined gene
18 sequence of the genome of the precursor cell to thereby
19 produce a desired cell wherein the desired non-selectable
20 gene sequence has been inserted into the predetermined gene
21 sequence; and wherein the presence or expression of the
22 introduced gene sequence in the cell of the recipient
23 comprises the gene therapy.

24 In particular, the invention includes the embodiments of
25 the above-stated method wherein the recipient is a non-
26 fungal plant, or a human or a non-human animal (particularly
27 a non-human animal is selected from the group consisting of:
28 a chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a
29 goat, a fish, a pig, a cow or bull, a non-human primate and
30 a human).

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1 The invention also provides a method for obtaining a
2 desired animal or non-fungal plant cell which contains a
3 desired non-selectable gene sequence inserted within a
4 predetermined gene sequence of the cell's genome, which
5 method comprises:

6 A. incubating a precursor cell under non-selective
7 culture conditions, or under a first set of selective
8 culture conditions, with a DNA molecule containing:

9 i) the desired non-selectable gene sequence,
10 wherein the DNA molecule additionally contains
11 two regions of homology which flank the desired
12 gene sequence, and which are sufficient to
13 permit the desired gene sequence to undergo
14 homologous recombination with the predetermined
15 gene sequence of the genome of the precursor
16 cell; and

17 ii) a selectable gene sequence whose presence or
18 expression in the cell can be selected for by
19 culturing the cells under the first set of
20 selective culture conditions, and whose
21 presence or expression in the cell can be
22 selected against by culturing the cells under
23 a second set of selective culture conditions;

24 B. permitting the DNA molecule to be introduced
25 into the precursor cell;

26 C. permitting the introduced DNA molecule to
27 undergo homologous recombination with the predetermined gene
28 sequence of the genome of the precursor cell to thereby
29 produce the desired cell wherein the desired non-selectable
30 gene sequence has been inserted into the predetermined gene
31 sequence; and

32 D. recovering the desired cell by culturing the
33 cell under the first set of selective culture conditions, by

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1 then permitting the cell to undergo intrachromosomal
2 recombination under non-selective culture conditions, and by
3 then incubating the cell under the second set of selective
4 culture conditions.

5 The invention also includes the embodiment wherein the
6 cell is deficient in an HPRT, APRT, or TK enzyme, and
7 wherein the selectable gene sequence expresses an active
8 HPRT, APRT, or TK enzyme, and wherein the first set of
9 selective culture conditions comprises incubation of the
10 cell under conditions in which the presence of an active
11 HPRT, APRT, or TK enzyme in the cell is required for growth,
12 and wherein the second set of selective culture conditions
13 comprises incubation of the cell under conditions in which
14 the absence of an active HPRT, APRT, or TK enzyme in the
15 cell is required for growth.
16
17

18 DESCRIPTION OF THE PREFERRED EMBODIMENTS:
19

20 The present invention concerns a method for introducing
21 DNA into the genome of a recipient plant or animal cell.
22 The method may be used to introduce such DNA into germ line
23 cells of animals (especially, rodents (i.e. mouse, rat,
24 hamster, etc.), rabbits, sheep, goats, fish, pigs, cattle
25 and non-human primates) in order to produce chimeric or
26 transgenic animals. The methods may also be used to
27 introduce DNA into plant cells which can then be manipulated
28 in order to produce chimeric or transgenic plants.

29 Alternatively, the method may be used to alter the
30 somatic cells of an animal (including humans) or a plant.
31 The plants and plant cells which may be manipulated through
32 application of the disclosed method include all
33 multicellular, higher (i.e. non-fungal or non-yeast) plants.

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1 I. Homologous R combination

2
3 The present invention provides a method for introducing
4 a desired gene sequence into a plant or animal cell. Thus,
5 it is capable of producing chimeric or transgenic plants and
6 animals having defined, and specific, gene alterations.

7 An understanding of the process of homologous
8 recombination (Watson, J.D., In: Molecular Biology of the
9 Gene, 3rd Ed., W.A. Benjamin, Inc., Menlo Park, CA (1977),
10 which reference is incorporated herein by reference) is
11 desirable in order to fully appreciate the present
12 invention.

13 In brief, homologous recombination is a well-studied
14 natural cellular process which results in the scission of
15 two nucleic acid molecules having identical or substantially
16 similar sequences (i.e. "homologous"), and the ligation of
17 the two molecules such that one region of each initially
18 present molecule is now ligated to a region of the other
19 initially present molecule (Sedivy, J.M., Bio-Technol.
20 6:1192-1196 (1988), which reference is incorporated herein
21 by reference).

22 Homologous recombination is, thus, a sequence specific
23 process by which cells can transfer a "region" of DNA from
24 one DNA molecule to another. As used herein, a "region" of
25 DNA is intended to generally refer to any nucleic acid
26 molecule. The region may be of any length from a singl
27 base to a substantial fragment of a chromosome.

28 For homologous recombination to occur between two DNA
29 molecules, the molecules must possess a "region of homology"
30 with respect to one another. Such a region of homology must
31 be at least two base pairs long. Two DNA molecules possess
32 such a "region of homology" when one contains a region whose

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1 sequence is so similar to a region in the second molecule
2 that homologous recombination can occur.

3 Recombination is catalyzed by enzymes which are
4 naturally present in both prokaryotic and eukaryotic cells.
5 The transfer of a region of DNA may be envisioned as
6 occurring through a multi-step process.

7 If either of the two participant molecules is a circular
8 molecule, then the above recombination event results in the
9 integration of the circular molecule into the other
10 participant.

11 Importantly, if a particular region is flanked by
12 regions of homology (which may be the same, but are
13 preferably different), then two recombinational events may
14 occur, and result in the exchange of a region of DNA between
15 two DNA molecules. Recombination may be "reciprocal," and
16 thus results in an exchange of DNA regions between two
17 recombining DNA molecules. Alternatively, it may be "non-
18 reciprocal," (also referred to as "gene conversion") and
19 result in both recombining nucleic acid molecules having the
20 same nucleotide sequence. There are no constraints
21 regarding the size or sequence of the region which is
22 exchanged in a two-event recombinational exchange.

23 The frequency of recombination between two DNA molecules
24 may be enhanced by treating the introduced DNA with agents
25 which stimulate recombination. Examples of such agents
26 include trimethylpsoralen, UV light, etc.
27

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1 II. Production of Chimeric and Transgenic Animals:
2 Gene Targeting Methods
3

4 One approach to producing animals having defined and
5 specific genetic alterations has used homologous
6 recombination to control the site of integration of an
7 introduced marker gene sequence in tumor cells and in
8 fusions between diploid human fibroblast and tetraploid
9 mouse erythroleukemia cells (Smithies, O. et al., Nature
10 317:230-234 (1985)).

11 This approach was further exploited by Thomas, K. R.,
12 and co-workers, who described a general method, known as
13 "gene targeting," for targeting mutations to a preselected,
14 desired gene sequence of an ES cell in order to produce a
15 transgenic animal (Mansour, S.L. et al., Nature 336:348-352
16 (1988); Capecchi, M.R. Trends Genet. 5:70-76 (1989);
17 Capecchi, M.R. et al., In: Current Communications in
18 Molecular Biology, Capecchi, M.R. (ed.), Cold Spring Harbor
19 Press, Cold Spring Harbor, NY (1989), pp. 45-52; which
20 references are incorporated herein by reference).

21 Gene targeting has been used to produce chimeric and
22 transgenic mice in which an nptII gene has been inserted
23 into the β_2 -microglobulin locus (Koller, B.H. et al., Proc.
24 Natl. Acad. Sci. (U.S.A.) 86:8932-8935 (1989); Zijlstra, M.
25 et al., Nature 342:435-438 (1989); Zijlstra, M. et al.,
26 Nature 344:742-746 (1989); DeChiaba et al., Nature 345:78-80
27 (1990)). Similar experiments have enabled the production of
28 chimeric and transgenic animals having a c-abl gene which
29 has been disrupted by the insertion of an nptII gene
30 (Schwartzberg, P.L. et al., Science 246:799-803 (1989)).
31 The technique has been used to produce chimeric mice in
32 which the en-2 gene has been disrupted by the insertion of

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1 an nptII gene (Joyner, A.L. et al., Nature 338:153-155
2 (1989)).

3 Gene targeting has also been used to correct an hprt
4 deficiency in an hprt ES cell line. Cells corrected of the
5 deficiency were used to produce chimeric animals.
6 Significantly, all of the corrected cells exhibited gross
7 disruption of the regions flanking the hprt locus; all of
8 the cells tested were found to contain at least one copy of
9 the vector used to correct the deficiency, integrated at the
10 hprt locus (Thompson, S. et al., Cell 56:313-321 (1989);
11 Koller, B.H. et al., Proc. Natl. Acad. Sci. (U.S.A.)
12 86:8927-8931 (1989)).

13 In order to utilize the "gene targeting" method, the
14 gene of interest must have been previously cloned, and the
15 intron-exon boundaries determined. The method results in
16 the insertion of a marker gene (i.e. the nptII gene) into a
17 translated region of a particular gene of interest. Thus,
18 use of the gene targeting method results in the gross
19 destruction of the gene of interest.

20 Recently, chimeric mice carrying the homeobox hox 1.1
21 allele have been produced using a modification of the gene
22 targeting method (Zimmer, A. et al., Nature 338:150-154
23 (1989). In this modification, the integration of vector
24 sequences was avoided by microinjecting ES cells with linear
25 DNA containing only a portion of the hox 1.1 allele, without
26 any accompanying vector sequences. The DNA was found to
27 cause the gene conversion of the cellular hox allele.
28 Selection was not used to facilitate the recovery of the
29 "converted" ES cells, which were identified using the
30 polymerase chain reaction ("PCR"). Approximately 50% of
31 cells which had been clonally purified from "converted"
32 cells were found to contain the introduced hox 1.1 allele,
33 suggesting to Zimmer, A. et al. either chromosomal

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1 instability or contamination of sample. None of the
2 chimeric mice were found to be able to transmit the
3 "converted" gene to their progeny (Zimmer, A. et al., In:
4 Current Communications in Molecular Biology, Capecchi, M.R.
5 (ed.), Cold Spring Harbor Press, Cold Spring Harbor, NY
6 (1989), pp. 53-58).

7 The use of the gene targeting method is illustrated in
8 Figure 1A. In that figure, a gene construct is produced in
9 which the nptII gene is inserted into an exon (designated
10 region "3") of a sequence of the hprt gene. The construct
11 is then permitted to undergo recombination with the hprt
12 gene of a cell. Such recombination results in the
13 replacement of the exon 3 sequence of the cell with the
14 disrupted exon 3 - nptII sequence of the construct.
15 Significantly, as illustrated in Figure 1A, the use of gene
16 targeting to alter a gene of a cell results in the formation
17 of a gross alteration in the sequence of that gene. As
18 indicated in Figure 1A, the efficiency of gene targeting is
19 approximately 1/300.

20
21 **III. Production of Chimeric and Transgenic Animals:**
22 **Use of Insertion Vectors**
23

24 In contrast to the above-described methods, the present
25 invention is capable of producing subtle, precise, and
26 predetermined mutations in the sequence of a desired gene of
27 a cell. The present invention has several embodiments, the
28 simplest of which is illustrated in Figure 1B.

29 As shown in Figure 1B, an insertion vector is used to
30 mutate the nucleotide sequence of the hprt gene. The use of
31 this vector type in combination with a second selectable
32 reversion event prevents the disruption of the chromosome by
33 the nptII gene or by the vector sequences. Thus, gross

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1 distortions of the recipient chromosome are avoided by the
2 present invention. Moreover, the efficiency of the gene
3 targeting was substantially improved (i.e. 1/32 as opposed
4 to 1/300).

5 The DNA molecule(s) which are to be introduced into the
6 recipient cell preferably contains a region of homology with
7 a region of the cellular genome. In a preferred embodiment,
8 the DNA molecule will contain two regions of homology with
9 the genome (both chromosomal and episomal) of the
10 pluripotent cell. These regions of homology will preferably
11 flank a "desired gene sequence" whose incorporation into the
12 cellular genome is desired. As stated above, the regions of
13 homology may be of any size greater than two bases long.
14 Most preferably, the regions of homology will be greater
15 than 10 bases long.

16 The DNA molecule(s) may be single stranded, but are
17 preferably double stranded. The DNA molecule(s) may be
18 introduced to the cell as one or more RNA molecules which
19 may be converted to DNA by reverse transcriptase or by other
20 means. Preferably, the DNA molecule will be double stranded
21 linear molecule. In the best mode for conducting the
22 invention, such a molecule is obtained by cleaving a closed
23 covalent circular molecule to form a linear molecule.
24 Preferably, a restriction endonuclease capable of cleaving
25 the molecule at a single site to produce either a blunt end
26 or staggered end linear molecule is employed. Most
27 preferably, the nucleotides on each side of this restriction
28 site will comprise at least a portion of the preferred two
29 regions of homology between the DNA molecule being
30 introduced and the cellular genome.

31 The invention thus provides a method for introducing the
32 "desired gene sequence" into the genome of an animal or
33 plant at a specific chromosomal location. The "desired gene

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sequence" may be of any length, and have any nucleotide sequence. It may comprise one or more gene sequences which encode complete proteins, fragments of such gene sequences, regulatory sequences, etc. Significantly, the desired gene sequence may differ only slightly from a native gene of the recipient cell (for example, it may contain single, or multiple base alterations, insertions or deletions relative to the native gene). The use of such desired gene sequences will permit one to create subtle and precise changes in the genome of the recipient cell. Thus, the present invention provides a means for manipulating and modulating gene expression and regulation.

In particular, the invention provides a mean for manipulating and modulating gene expression and protein structure through the replacement of a gene sequence with a "non-selectable" "desired gene sequence." A gene sequence is non-selectable if its presence or expression in a recipient cell provides no survival advantage to the cell under the culturing conditions employed. Thus, by definition, one cannot select for cells which have received a "non-selectable" gene sequence. In contrast, a "dominant" gene sequence is one which can under certain circumstances provide a survival advantage to a recipient cell. The neomycin resistance conferred by the nptII gene is a survival advantage to a cell cultured in the presence of neomycin or G418. The nptII gene is thus a dominant, rather than a non-selectable gene sequence.

In particular, the invention permits the replacement of a gene sequence which is present in the recipient cell with an "analog" sequence. A sequence is said to be an analog of another sequence if the two sequences are substantially similar in sequence, but have minor changes in sequence corresponding to single base substitutions, deletions, or

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1 insertions with respect to one another, or if they possess
2 "minor" multiple base alterations. Such alterations are
3 intended to exclude insertions of dominant selectable marker
4 genes.

5 When the desired gene sequence, flanked by regions of
6 homology with the recipient cell, is introduced into the
7 recipient cell as a linear double stranded molecule, whose
8 termini correspond to the regions of homology, a single
9 recombination event with the cell's genome will occur in
10 approximately 5% of the transfected cells. Such a single
11 recombinational event will lead to the integration of the
12 entire linear molecule into the genome of the recipient
13 cell.

14 The structure generated by the integration of the linear
15 molecule will undergo a subsequent, second recombinational
16 event (approximately 10^{-5} - 10^{-7} per cell generation). This
17 second recombinational event will result in the elimination
18 of all DNA except for the flanking regions of homology, and
19 the desired DNA sequence from the integrated structure.

20 Thus, the consequence of the second recombinational event
21 is to replace the DNA sequence which is normally present
22 between the flanking regions of homology in the cell's
23 genome, with the desired DNA sequence, and to eliminate the
24 instability of gene replacement.

25 The DNA molecule containing the desired gene sequence
26 may be introduced into the pluripotent cell by any method
27 which will permit the introduced molecule to undergo
28 recombination at its regions of homology. Some methods,
29 such as direct microinjection, or calcium phosphate
30 transformation, may cause the introduced molecule to form
31 concatemers upon integration. These concatemers may resolve
32 themselves to form non-concatemeric integration structures.
33 Since the presence of concatemers is not desired, methods

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1 which produce them are not preferred. In a preferred
2 embodiment, the DNA is introduced by electroporation
3 (Toneguzzo, F. et al., Nucleic Acids Res. 16:5515-5532
4 (1988); Quillet, A. et al., J. Immunol. 141:17-20 (1988);
5 Machy, P. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:8027-
6 8031 (1988); all of which references are incorporated herein
7 by reference).

8 After permitting the introduction of the DNA
9 molecule(s), the cells are cultured under conventional
10 conditions, as are known in the art.

11 In order to facilitate the recovery of those cells which
12 have received the DNA molecule containing the desired gene
13 sequence, it is preferable to introduce the DNA containing
14 the desired gene sequence in combination with a second gene
15 sequence which would contain a detectable marker gene
16 sequence. For the purposes of the present invention, any
17 gene sequence whose presence in a cell permits one to
18 recognize and clonally isolate the cell may be employed as
19 a detectable marker gene sequence.

20 In one embodiment, the presence of the detectable marker
21 sequence in a recipient cell is recognized by hybridization,
22 by detection of radiolabelled nucleotides, or by other
23 assays of detection which do not require the expression of
24 the detectable marker sequence. Preferably, such sequences
25 are detected using PCR (Mullis, K. et al., Cold Spring
26 Harbor Symp. Quant. Biol. 51:263-273 (1986); Erlich H. et
27 al., EP 50,424; EP 84,796, EP 258,017, EP 237,362; Mullis,
28 K., EP 201,184; Mullis K. et al., US 4,683,202; Erlich, H.,
29 US 4,582,788; and Saiki, R. et al., US 4,683,194), which
30 references are incorporated herein by reference).

31 PCR achieves the amplification of a specific nucleic
32 acid sequence using two oligonucleotide primers
33 complementary to regions of the sequence to be amplified.

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1 Extension products incorporating the primers then become
2 templates for subsequent replication steps. PCR provides a
3 method for selectively increasing the concentration of a
4 nucleic acid molecule having a particular sequence even when
5 that molecule has not been previously purified and is
6 present only in a single copy in a particular sample. The
7 method can be used to amplify either single or double
8 stranded DNA.

9 Most preferably, however, the detectable marker gene
10 sequence will be expressed in the recipient cell, and will
11 result in a selectable phenotype. Examples of such
12 preferred detectable gene sequences include the hprt gene
13 (Littlefield, J.W., Science 145:709-710 (1964), herein
14 incorporated by reference), a xanthine-guanine
15 phosphoribosyltransferase (gpt) gene, or an adenosine
16 phosphoribosyltransferase (aprt) gene (Sambrook et al., In:
17 Molecular Cloning A Laboratory Manual, 2nd. Ed., Cold Spring
18 Harbor Laboratory Press, NY (1989), herein incorporated by
19 reference), a tk gene (i.e. thymidine kinase gene) and
20 especially the tk gene of herpes simplex virus (Giphart-
21 Gassler, M. et al., Mutat. Res. 214:223-232 (1989) herein
22 incorporated by reference), the nptII gene (Thomas, K.R. et
23 al., Cell 51:503-512 (1987); Mansour, S.L. et al., Nature
24 336:348-352 (1988), both references herein incorporated by
25 reference), or other genes which confer resistance to amino
26 acid or nucleoside analogues, or antibiotics, etc. Examples
27 of such genes include gene sequences which encode enzymes
28 such as dihydrofolate reductase (DHFR) enzyme, adenosine
29 deaminase (ADA), asparagine synthetase (AS), hygromycin B
30 phosphotransferase, or a CAD enzyme (carbamyl phosphate
31 synthetase, aspartate transcarbamylase, and dihydroorotase)
32 (Sambrook et al., In: Molecular Cloning A Laboratory Manual,

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1 2nd. Ed., Cold Spring Harbor Laboratory Press, NY (1989),
2 herein incorporated by reference).

3 Cells that do not contain an active thymidine kinase
4 (TK) enzyme, a hypoxanthine-phosphoribosyltransferase (HPRT)
5 enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)
6 enzyme, or an adenosine phosphoribosyltransferase (APRT)
7 enzyme, are unable to grow in medium containing
8 hypoxanthine, aminopterin, and/or mycophenolic acid (and
9 preferably adenine, xanthine, and/or thymidine), and
10 thymidine, but are able to grow in medium containing
11 nucleoside analogs such as 5-bromodeoxyuridine, 6-
12 thioguanine, 8-azapurine, etc. (Littlefield, J.W., Science
13 145:709-710 (1964); Sambrook et al., In: Molecular Cloning
14 A Laboratory Manual, 2nd. Ed., Cold Spring Harbor Laboratory
15 Press, NY (1989)).

16 Conversely, cells that do contain such active enzymes
17 are able to grow in such medium, but are unable to grow in
18 medium containing nucleoside analogs such as 5-
19 bromodeoxyuridine, 6-thioguanine, 8-azapurine, etc.
20 (Sambrook et al., In: Molecular Cloning A Laboratory Manual,
21 2nd. Ed., Cold Spring Harbor Laboratory Press, NY (1989)).

22 Cells expressing active thymidine kinase are able to
23 grow in media containing HATG, but are unable to grow in
24 media containing nucleoside analogues such as 5-azacytidine
25 (Giphart-Gassler, M. et al., Mutat. Res. 214:223-232
26 (1989)). Cells containing an active HSV-tk gene are
27 incapable of growing in the presence of gangcylovir or
28 similar agents.

29 The detectable marker gene may be any gene which can
30 complement for a recognizable cellular deficiency. Thus,
31 for example, the gene for HPRT could be used as the
32 detectable marker gene sequence when employing cells lacking
33 HPRT activity. Thus, this gene is an example of a gene

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1 whose expression product may be used to select mutant cells,
2 or to "negatively select" for cells which express this gene
3 product.

4 The nptII gene (Southern, P.J., et al., J. Molec. Appl.
5 Genet. 1:327-341 (1982); Smithies, O. et al., Nature
6 317:230-234 (1985), which references are incorporated herein
7 by reference) is the most preferred detectable marker gene
8 sequence. Constructs which contain both an nptII gene and
9 either a tk gene or an hprt gene are especially preferred.
10

11 A. Use of a Single DNA Molecule Containing Both the
12 Detectable Marker Sequence and the Desired Gen
13 Sequence
14

15 In a first preferred embodiment, the detectable marker
16 gene sequence, flanked by the regions of homology, is
17 provided to the recipient cells on the same DNA molecule
18 which contains the desired gene sequence. As discussed
19 previously, it is preferred that this DNA molecule be a
20 linear molecule.

21 After selection for cells which have incorporated the
22 desired DNA molecule (for example by selection for G418
23 resistant cells when the detectable marker gene sequence is
24 an expressible nptII gene sequence), the cells are cultured,
25 and the presence of the introduced DNA molecule is confirmed
26 as described above. Approximately 10^7 cells are cultured and
27 screened for cells which have undergone the second
28 recombinational event (discussed above) resulting in the
29 replacement of a native sequence (i.e. a gene sequence
30 which is normally and naturally present in the recipient
31 cell) with the desired gene sequence.

32 Any of a variety of methods may be used to identify
33 cells which have undergone the second recombinational event.

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1 Direct screening of clones, use of PCR, use of hybridization
2 probes, etc., may all be employed for this purpose. In a
3 preferred embodiment, the DNA molecule will, in addition to
4 the desired gene sequence, the flanking regions of homology
5 and the detectable marker gene sequence, contain an
6 additional gene sequence which will permit the selection or
7 recognition of cells which have undergone the second
8 recombinational event. This additional gene sequence will
9 be excised from the cell's genome as a direct consequence of
10 the second recombinational event. Thus, gene sequences
11 which are suitable for this purpose include any gene
12 sequence whose loss from a cell can be detected or selected
13 for. Examples of such "negative selection" gene sequences
14 include the hprt gene, and the tk gene (especially the tk
15 gene of herpes simplex virus).

16 In the first preferred embodiment, the frequency of the
17 second recombinational event is approximately 10^{-5} . However,
18 the use of a "negative selection" gene sequence permits one
19 to identify such recombinant cells at a frequency of
20 approximately 100%.

21 As illustrated in Figure 2, the DNA molecule may have a
22 region of heterology located at the proposed insertion site.
23 Insertion of such a vector permits one to select for
24 recombinants which have recombined at the insertion site
25 (and not at other potential sites). If recombination occurs
26 at the desired insertion site, it will lead to the loss of
27 the sequence of heterology located at the proposed insertion
28 site of the DNA molecule (HSVtk, for example, in Figure 2A).
29 Insertions which result from other recombinational events
30 will retain the sequence of heterology. Thus, by employing
31 a region of heterology which encodes an assayable gene
32 product, or which can be used as a "negative selectable"
33 marker, one can readily determine that the locus of

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1 insertion of the recipient cell contains the precise
2 sequence desired. As indicated in Figure 2A), the
3 efficiency of such a vector is approximately 1/197.

4 The region of heterology which may be introduced at the
5 insertion site of the DNA molecule may be either short (for
6 example, 26 base pairs, Figure 2B) or of substantial size
7 (for example, 2 kb, Figure 2A). The site of linearization
8 may be 5', 3', or within the region of heterology. When the
9 site of linearization is within the region of heterology,
10 the efficiency of gene targeting is 1/63.

11 As shown in Figure 2C, the region of heterology may be
12 located at a site internal to the region of homology where
13 the desired recombination shall occur. Such a construct can
14 be used when one desires to introduce a subtle mutation into
15 a locus of the cellular gene at a site other than that of
16 the site of desired recombination.

17
18 **B. Use of a Different DNA Molecules to Provide the**
19 **Detectable Marker Sequence and the Desired Gen**
20 **Sequence**

21
22 In a second preferred embodiment, the detectable marker
23 gene sequence, flanked by the regions of homology, will be
24 provided to the recipient cell on a different DNA molecule
25 from that which contains the desired gene sequence. It is
26 preferred that these molecules be linear molecules.

27 When provided on separate DNA molecules, the detectable
28 marker gene sequence and the desired gene sequence will most
29 preferably be provided to the recipient cell by co-
30 electroporation, or by other equivalent techniques.

31 After selection of such recipients (preferably through
32 the use of a detectable marker sequence which expresses the
33 nptII gene and thus confers cellular resistance to the

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1 antibiotic G418), the cells are grown up and screened to
2 confirm the insertion event (preferably using PCR).

3 In the absence of any selection, only one cell in 10^7
4 would be expected to have the predicted recombinant
5 structures. If, however, one selects for recipient cells
6 which contain and express a detectable marker sequence (such
7 as the nptII gene), it is possible to obtain a 10^3 to 10^5
8 fold enrichment for cells which have taken up both DNA
9 molecules. Typically, such enrichment enables one to
10 identify the desired recipient cell (in which the introduced
11 DNA has integrated into the cell's genome) by screening only
12 800 -1,500 cells. Such screening is preferably done using
13 PCR, or other equivalent methods. Using such negative
14 selection techniques, one may manipulate the vector copy
15 number.

16 The two introduced DNA molecules will generally not have
17 integrated into the same site in the genome of the recipient
18 cell. Thus, in some cases, the desired gene sequence will
19 have integrated in a manner so as to replace the native
20 cellular gene sequence between the flanking regions of
21 homology. The locus of integration of the detectable marker
22 gene is unimportant for the purposes of the present
23 invention, provided it is not genetically linked to the same
24 locus as the desired gene sequence. If desired, however, it
25 is possible to incorporate a gene sequence capable of
26 negative selection along with the DNA containing the
27 detectable marker sequence. Thus, one can ultimately select
28 for cells which have lost the introduced selectable marker
29 gene sequence DNA.
30

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C. Use of Direct Selection to Identify Homologous Recombination Events

Although all of the above-described preferred embodiments enable the isolation of cells in which one of a cell's alleles has been mutated to contain a desired gene sequence, each embodiment requires the screening of a significant number of candidate cells in order to identify the desired recombinant cell. It is, however, possible to directly select for the desired recombinant cell by employing a variation of the above embodiments. This embodiment of the invention is illustrated in Figure 13. In the methods illustrated in Figure 13, if the sequence located below the asterisk is a neo gene, then only the mutant revertants will be selected if 6-thioguanine and G418 selection is applied to select for the excision events.

The method for direct selection of the desired cells relies upon the phenotypic difference in targeted and non-targeted cells and the use of a single gene which can be used for both positive and negative selection.

Typically, in any homologous recombination experiment performed with an insertion vector, three populations of cells will be created. The first class of cells will be those which have failed to receive the desired DNA molecule. This class will comprise virtually all of the candidate cells isolated on completion of the experiment. The second class of cells will be those cells in which the desired gene sequence has been incorporated at a random insertion site (i.e. a site other than in the gene desired to be mutated). Approximately one cell in 10^3 - 10^4 total cells will be in this class. The third class of cells will be those cells in which the desired gene sequence has been incorporated by homologous recombination into a site in the desired gene.

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1 Approximately one cell in 10^5 - 10^6 total cells will be in this
2 class.

3 In the above-described embodiments, the cells of the
4 first class (non-transfected cells) can be eliminated by
5 positive selection, thus necessitating the screening of only
6 about 1,000 cells in order to identify the desired
7 recombinant cell. In the present embodiment, cells of the
8 third class (homologous recombinants) may be selected from
9 the cells of the second class (random insertions) if a
10 phenotypic difference exists between the cells of the two
11 classes.

12 Since random integration sites are likely to be
13 concatemeric with few single copy clones (depending upon the
14 DNA concentration with which the cells were transfected),
15 such integration events are inherently unstable. Thus, such
16 concatemeric constructs will typically undergo intrachromo-
17 somal recombination. Such recombination will always leave
18 one intact copy of the vector in the genome. Thus, all
19 random insertion events may be negatively selected from the
20 population if a negatively selectable marker is included on
21 the vector.

22 In contrast, cells in which the desired gene sequence
23 has been incorporated into the desired gene by homologous
24 recombination will revert with a relatively high frequency
25 (approximately 1 in 10^4 - 10^5 per cell division (depending upon
26 the size of the duplicated structure) to produce a mutated
27 desired gene that does not contain vector sequences.
28 Therefore, even if the vector contained a negatively
29 selectable gene sequence, such cells will survive negative
30 selection, and can be recovered. The majority of homologous
31 recombinant cells do not undergo reversion, and will be
32 eliminated by the negative selection. Thus, the sum of the

1 selections will result in the isolation of the desired
2 recombinants.

3 The method comprises incubating a "precursor cell" (i.e.
4 a cell which is to be changed by application of the method
5 into the "desired" recombinant cell) under non-selective
6 culture conditions, or under a first set of selective
7 culture conditions. A culturing condition (i.e. medium,
8 temperature, etc.) is said to be "non-selective" if it is
9 capable of promoting the growth (or sustaining the
10 viability) of a precursor cell, a desired cell, and an
11 intermediate cell type (i.e. a cell obtained during the
12 progression of a precursor cell into a desired cell). A
13 culturing condition is said to be "selective" if it is
14 capable of promoting the growth (or sustaining the
15 viability) of only certain cells (i.e. those having a
16 particular genotype and which therefore contain a particular
17 gene product in either an active or an inactive form).

18 Preferred selective culturing conditions thus depend
19 upon the genotype of the precursor cell. As stated above,
20 cells that do not contain an active thymidine kinase (TK)
21 enzyme, a hypoxanthine-phosphoribosyltransferase (HPRT)
22 enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)
23 enzyme, or an adenosine phosphoribosyltransferase (APRT)
24 enzyme, are unable to grow in medium containing
25 hypoxanthine, aminopterin, and/or mycophenolic acid (and
26 preferably adenine, xanthine, and/or thymidine), and
27 thymidine, but are able to grow in medium containing
28 nucleoside analogs such as 5-bromodeoxyuridine, 6-
29 thioguanine, 8-azapurine, etc. Conversely, cells that do
30 contain such active enzymes are able to grow in such medium,
31 but are unable to grow in medium containing nucleoside
32 analogs such as 5-bromodeoxyuridine, 6-thioguanine, 8-
33 azapurine, etc.

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1 Such incubation is conducted in the presence of a DNA
2 molecule containing a desired non-selectable gene sequence.
3 Preferably, the DNA molecule additionally contains two
4 regions of homology which flank this desired gene sequence,
5 and which are sufficient to permit the desired gene sequence
6 to undergo homologous recombination with a predetermined
7 gene sequence of the genome of the precursor cell. The DNA
8 molecule additionally contains a selectable gene sequence
9 whose presence or expression in the cell can be selected for
10 by culturing the cell under a first set of selective culture
11 conditions, and whose presence or expression in the cell can
12 be selected against by culturing the cell under a second set
13 of selective culture conditions.

14 Examples of preferred selectable gene sequences include
15 gene sequences which encode an active thymidine kinase (TK)
16 enzyme, a hypoxanthine-phosphoribosyltransferase (HPRT)
17 enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)
18 enzyme, or an adenosine phosphoribosyltransferase (APRT)
19 enzyme. Such gene sequences can be used for both positive
20 and negative selection.

21 Additional gene sequences which can be used as
22 selectable gene sequences include those which encode enzymes
23 such as dihydrofolate reductase (DHFR) enzyme, adenosine
24 deaminase (ADA), asparagine synthetase (AS), hygromycin B
25 phosphotransferase, or a CAD enzyme (carbamyl phosphate
26 synthetase, aspartate transcarbamylase, and dihydroorotase).
27 Methods for producing cells deficient in expressing these
28 enzymes are described by Sambrook et al. (In: Molecular
29 Cloning A Laboratory Manual, 2nd. Ed., Cold Spring Harbor
30 Laboratory Press, NY (1989), herein incorporated by
31 reference). Such gene sequences can be used only for
32 positive selection.

1 The incubation is performed under conditions sufficient
2 to permit the DNA molecule to be introduced into the
3 precursor cell. Such introduced DNA molecules are able to
4 then undergo homologous recombination with the predetermined
5 gene sequence of the genome of the precursor cell to thereby
6 produce the desired cell wherein the desired non-selectable
7 gene sequence has been inserted into the predetermined gene
8 sequence.

9 Such a desired cell can be recovered by culturing the
10 cell under the first set of selective culture conditions, by
11 then permitting the cell to undergo intrachromosomal
12 recombination under non-selective culture conditions, and by
13 then incubating the cell under the second set of selective
14 culture conditions.

15 Thus, in one preferred embodiment, the precursor cell
16 lacks an active hypoxanthine-phosphoribosyltransferase (HPRT)
17 enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)
18 enzyme, or an adenosine phosphoribosyltransferase (APRT)
19 enzyme, and the selectable gene sequence expresses an active
20 HPRT, XGPRT or APRT enzyme. In the first set of selectable
21 culture conditions, medium containing hypoxanthine,
22 aminopterin and/or mycophenolic acid (and preferably
23 adenine, xanthine, and/or thymidine) is employed. In the
24 second set of selectable culturing conditions, medium
25 containing a nucleoside analog such as 5-bromodeoxyuridine,
26 6-thioguanine, 8-azapurine, etc., is employed.

27 In a second preferred embodiment, the precursor cell
28 lacks an active TK enzyme, and the selectable gene sequence
29 expresses an active TK enzyme. In the first set of
30 selectable culture conditions, medium containing
31 hypoxanthine, aminopterin, and thymidine is employed. In
32 the second set of selectable culturing conditions, medium
33 containing a thymidine analog such as FIAU (Borrelli, Proc.

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1 Nat'l. Acad. Sci. (U.S.A.) 85:7572 (1988), or gangcyclovir,
2 etc. is employed (if an HSV tk gene is used), or 5-
3 bromodeoxyuridine, etc. (if a cellular tk gene is employed).

4 A preferred negative selectable marker is the hprt gene
5 (cells expressing an active HPRT enzyme are unable to grow
6 in the presence of certain nucleoside analogues such as 6-
7 thioguanine, etc.). When using 6-thioguanine as a negative
8 selection agent, a density of 10^4 cells / cm^2 is preferably
9 used since the efficiency of 6-thioguanine selection is cell
10 density dependent. A typical experiment with 10^7 transfected
11 cells would yield approximately 10 revertant cells after
12 successive selection. The relative yield of revertant
13 clones can be substantially increased by using "Poly A
14 Selection" for the first round of selection. "Poly A
15 Selection" is discussed in detail in Example 6 below.

16 17 IV. The Production of Chimeric and Transgenic Animals

18
19 The chimeric or transgenic animals of the present
20 invention are prepared by introducing one or more DNA
21 molecules into a precursor pluripotent cell, most preferably
22 an ES cell, or equivalent (Robertson, E.J., In: Current
23 Communications in Molecular Biology, Capecchi, M.R. (ed.),
24 Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), pp.
25 39-44, which reference is incorporated herein by reference).
26 The term "precursor" is intended to denote only that the
27 pluripotent cell is a precursor to the desired
28 ("transfected") pluripotent cell which is prepared in
29 accordance with the teachings of the present invention. The
30 pluripotent (precursor or transfected) cell may be cultured
31 in vivo, in a manner known in the art (Evans, M.J. et al.,
32 Nature 292:154-156 (1981)) to form a chimeric or transgenic
33 animal.

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1 Any ES cell may be used in accordance with the present
2 invention. It is, however, preferred to use primary
3 isolates of ES cells. Such isolates may be obtained
4 directly from embryos such as the CCE cell line disclosed by
5 Robertson, E.J., In: Current Communications in Molecular
6 Biology, Capecchi, M.R. (ed.), Cold Spring Harbor Press,
7 Cold Spring Harbor, NY (1989), pp. 39-44), or from the
8 clonal isolation of ES cells from the CCE cell line
9 (Schwartzberg, P.A. et al., Science 246:799-803 (1989),
10 which reference is incorporated herein by reference). Such
11 clonal isolation may be accomplished according to the method
12 of E.J. Robertson (In: Teratocarcinomas and Embryonic Stem
13 Cells: A Practical Approach, (E.J. Robertson, Ed.), IRL
14 Press, Oxford, 1987) which reference and method are
15 incorporated herein by reference. The purpose of such
16 clonal propagation is to obtain ES cells which have a
17 greater efficiency for differentiating into an animal.
18 Clonally selected ES cells are approximately 10-fold more
19 effective in producing transgenic animals than the
20 progenitor cell line CCE. For the purposes of the
21 recombination methods of the present invention, clonal
22 selection provides no advantage. An example of ES cell
23 lines which have been clonally derived from embryos are the
24 ES cell lines, AB1 (hprt⁺) or AB2.1 (hprt⁻).

25 The ES cells are preferably cultured on stomal cells
26 (such as STO cells (especially SNC4 STO cells) and/or
27 primary embryonic fibroblast cells) as described by E.J.
28 Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A
29 Practical Approach, (E.J. Robertson, Ed.), IRL Press,
30 Oxford, 1987, pp 71-112), which reference is incorporated
31 herein by reference. The stomal (and/or fibroblast) cells
32 serve to eliminate the clonal overgrowth of abnormal ES
33 cells. Most preferably, the cells are cultured in the

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1 presence of leukocyte inhibitory factor ("lif") (Gough, N.M.
2 et al., Reprod. Fertil. Dev. 1:281-288 (1989); Yamamori, Y.
3 et al., Science 246:1412-1416 (1989), both of which
4 references are incorporated herein by reference). Since the
5 gene encoding lif has been cloned (Gough, N.M. et al.,
6 Reprod. Fertil. Dev. 1:281-288 (1989)), it is especially
7 preferred to transform stomal cells with this gene, by means
8 known in the art, and to then culture the ES cells on
9 transformed stomal cells that secrete lif into the culture
10 medium.

11 ES cell lines may be derived or isolated from any
12 species (for example, chicken, etc.), although cells derived
13 or isolated from mammals such as rodents (i.e. mouse, rat,
14 hamster, etc.), rabbits, sheep, goats, fish, pigs, cattle,
15 primates and humans are preferred.

16 17 V. The Production of Chimeric and Transgenic Plants

18
19 The chimeric or transgenic plants of the invention are
20 produced through the regeneration of a plant cell which has
21 received a DNA molecule through the use of the methods
22 disclosed herein.

23 All plants from which protoplasts can be isolated and
24 cultured to give whole regenerated plants can be transformed
25 by the present invention so that whole plants are recovered
26 which contain the introduced gene sequence. Some suitable
27 plants include, for example, species from the genera
28 Fragaria, Lotus, Medicago, Onobrychis, Trifolium,
29 Trigonella, Vigna, Citrus, Linum, Geranium, Manicot, Daucus,
30 Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum,
31 Datura, Hyoscyamus, Lycopersion, Nicotiana, Solanum,
32 Petunia, Digitalis, Majorana, Cichorium, Helianthus,
33 Lactuca, Bromus, Asparagus, Antirrhinum, Hemerocallis,

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1 Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus,
2 Senecio, Salpiglossis, Cucumis, Browallia, Glycine, Lolium,
3 Zea, Triticum, Sorghum, Ipomoea, Passiflora, Cyclamen,
4 Malus, Prunus, Rosa, Rubus, Populus, Santalum, Allium,
5 Lilium, Narcissus, Ananas, Arachis, Phaseolus, Pisum and
6 Datura.

7 There is an increasing body of evidence that practically
8 all plants can be regenerated from cultured cells or
9 tissues, including but not limited to all major cereal crop
10 species, sugarcane, sugar beet, cotton, fruit and other
11 trees, legumes and vegetables.

12 Plant regeneration from cultural protoplasts is
13 described in Evans et al., "Protoplast Isolation and
14 Culture," in Handbook of Plant Cell Culture 1:124-176
15 (MacMillan Publishing Co., New York, 1983); M.R. Davey,
16 "Recent Developments in the Culture and Regeneration of
17 Plant Protoplasts," Protoplasts, 1983 - Lecture Proceedings,
18 pp. 19-29 (Birkhauser, Basel, 1983); P.J. Dale, "Protoplast
19 Culture and Plant Regeneration of Cereals and Other
20 Recalcitrant Crops," in Protoplasts 1983 - Lecture
21 Proceedings, pp. 31-41 (Birkhauser, Basel, 1983); and H.
22 Binding, "Regeneration of Plants," in Plant Protoplasts, pp.
23 21-37 (CRC Press, Boca Raton, 1985).

24 Regeneration varies from species to species of plants,
25 but generally a suspension of transformed protoplasts
26 containing the introduced gene sequence is formed. Embryo
27 formation can then be induced from the protoplast
28 suspensions, to the stage of ripening and germination as
29 natural embryos. The culture media will generally contain
30 various amino acids and hormones, such as auxin and
31 cytokinins. It is also advantageous to add glutamic acid
32 and proline to the medium, especially for such species as
33 corn and alfalfa. Shoots and roots normally develop

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1 simultaneously. Efficient regeneration will depend on the
2 medium, on the genotype, and on the history of the culture.
3 If these three variables are controlled, then regeneration
4 is fully reproducible and repeatable.

5 The mature plants, grown from the transformed plant
6 cells, are selfed to produce an inbred plant. The inbred
7 plant produces seed containing the introduced gene sequence.
8 These seeds can be grown to produce plants that express this
9 desired gene sequence.

10 Parts obtained from the regenerated plant, such as
11 flowers, seeds, leaves, branches, fruit, and the like are
12 covered by the invention. Progeny and variants, and mutants
13 of the regenerated plants are also included within the scope
14 of this invention.

15 As used herein, variant describes phenotypic changes
16 that are stable and heritable, including heritable variation
17 that is sexually transmitted to progeny of plants.
18

19 VI. GENE EXPRESSION

20
21 In one embodiment, the DNA molecule(s) which are to be
22 introduced into the recipient cells in accordance with the
23 methods of the present invention will be incorporated into
24 a plasmid or viral vector (or a derivative thereof) capable
25 of autonomous replication in a host cell.

26 Preferred prokaryotic vectors include plasmids such as
27 those capable of replication in E. coli such as, for
28 example, pBR322, ColE1, pSC101, pACYC 184, π VX. Such
29 plasmids are, for example, disclosed by Maniatis, T., et al.
30 (In: Molecular Cloning, A Laboratory Manual, Cold Spring
31 Harbor Press, Cold Spring Harbor, NY (1982)). Bacillus
32 plasmids include pC194, pC221, pT127, etc. Such plasmids
33 are disclosed by Gryczan, T. (In: The Molecular Biology of

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1 the Bacilli, Academic Press, NY (1982), pp. 307-329).
2 Suitable Streptomyces plasmids include pIJ101 (Kendall,
3 K.J., et al., J. Bacteriol. 169:4177-4183 (1987)), and
4 Streptomyces bacteriophages such as ϕ C31 (Chater, K.F., et
5 al., In: Sixth International Symposium on Actinomycetales
6 Biology, Akademiai Kiado, Budapest, Hungary (1986), pp. 45-
7 54). Pseudomonas plasmids are reviewed by John, J.F., et
8 al. (Rev. Infect. Dis. 8:693-704 (1986)), and Izaki, K.
9 (Jpn. J. Bacteriol. 33:729-742 (1978)).

10 Examples of suitable yeast vectors include the yeast 2-
11 micron circle, the expression plasmids YEP13, YCP and YRP,
12 etc., or their derivatives. Such plasmids are well known in
13 the art (Botstein, D., et al., Miami Wntr. Symp. 19:265-274
14 (1982); Broach, J.R., In: The Molecular Biology of the
15 Yeast Saccharomyces: Life Cycle and Inheritance, Cold
16 Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470
17 (1981); Broach, J.R., Cell 28:203-204 (1982)).

18 Examples of vectors which may be used to replicate the
19 DNA molecules in a mammalian host include animal viruses
20 such as bovine papilloma virus, polyoma virus, adenovirus,
21 or SV40 virus.

22 VII. Uses of the Present Invention

23 The methods of the present invention permit the
24 introduction of a desired gene sequence into an animal or
25 plant cell.

26 In a first embodiment, the methods of the present
27 invention may be used to introduce DNA into germ line cells
28 of animals in order to produce chimeric or transgenic
29 animals which contain a desired gene sequence. The animals
30 which may be produced through application of the described
31 method include chicken, non-human mammals (especially,
32
33

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1 rodents (i.e. mouse, rat, hamster, etc.), rabbits, sheep,
2 goats, fish, pigs, cattle and non-human primates).

3 As stated above, the desired gene sequence may be of any
4 length, and have any nucleotide sequence. In particular, it
5 is possible to design the sequence of the desired gene
6 sequence in order to create single, or multiple base
7 alterations, insertions or deletions in any preselected gene
8 of a cell.

9 If such changes are within a translated region of a
10 native gene sequence, then a new protein variant of a native
11 protein can be obtained. Such a procedure can, for example
12 be used to produce animals which produce improved (i.e. more
13 stable, more active, etc.) enzymes, binding proteins,
14 receptors, receptor ligands, etc.

15 The methods of the present invention may be used to
16 produce cells in which a natural gene has been replaced with
17 a heterologous gene. A gene is said to be heterologous to
18 a transgenic cell if it is derivable from a species other
19 than that of the transgenic cell.

20 In one embodiment, this replacement may be accomplished
21 in a single step (Figure 3). To accomplish such
22 replacement, a DNA molecule containing a desired gene
23 sequence and a region of homology is introduced into a
24 recipient cell. A selectable marker gene is also introduced
25 into the cell, and used to select for cells which have
26 underwent recombination. The method results in the
27 replacement of the normal sequences adjacent to the region
28 of homology with the heterologous sequences of the desired
29 DNA sequence.

30 In a second embodiment, this replacement may be
31 accomplished in a two steps (Figure 4). As in the
32 embodiment described above, a cell is provided with a DNA
33 molecule containing a desired gene sequence and a region of

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1 homology. The DNA molecule also contains a selectable
2 marker gene used to select for cells which have undergone a
3 recombinational event that has resulted in the insertion of
4 the introduced DNA molecule into their chromosomes at the
5 site of homology. The structure of such an insertion site
6 is depicted in Figure 4A.

7 Significantly, in this embodiment, the introduced DNA
8 molecule will also contain a "negative selectable" marker
9 gene which can be used to select for cells which undergo a
10 second recombinational event that results in the loss of the
11 inserted DNA.

12 As shown in Figure 4B, a second DNA molecule is employed
13 to complete the gene replacement. This second DNA molecule
14 need not contain any selectable marker gene. Upon receipt
15 of the second DNA molecule, a second recombinational event
16 occurs which exchanges the "second" DNA molecule for the
17 integrated "first" DNA molecule (including the desired DNA
18 sequence, the selectable marker sequence, and the "negative
19 selectable" marker sequence contained on that molecule).
20 This aspect of the invention is illustrated in Figure 4B.

21 In another embodiment of the invention, subtle mutations
22 may be introduced into a desired locus using a "cassette"
23 construct containing both a positive selection marker (such
24 as the nptII gene or the gpt gene) and a negative selection
25 marker (such as the tk gene). In this embodiment, one first
26 uses the positive selection capacity of the construct to
27 introduce the two selection markers into a desired locus.
28 One then introduces the desired subtle mutations (substi-
29 tutions, insertions, deletions, etc.) by providing a cell
30 with a DNA molecule that contains the desired mutation. By
31 selecting for the loss of the "cassette" (using the negative
32 selection marker), one can select for recombinational events
33 which result in the replacement of the "cassette" sequence

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1 with the DNA sequence containing the desired mutation. This
2 embodiment of the invention is illustrated in Figure 5.

3 The methods of the present invention may also be used to
4 replace contiguous regions of a chromosome with any desired
5 gene sequence. Thus, the present invention is not limited
6 in the size of the DNA regions which may be altered or
7 replaced. This aspect of the present invention is
8 illustrated in Figure 6, as a series of 5 steps (Figures 6A-
9 6E). The method is applicable to any gene sequence. It is
10 especially useful in producing cells which contain
11 heterologous immunoglobulins (such as the heavy chain locus
12 of an immunoglobulin).

13 The first step in replacing a large region of a
14 chromosome with a desired sequence involves setting up an
15 initial target. In this step, a recipient cell is provided
16 with a DNA molecule which contains a "first fragment" of the
17 total desired replacement sequence (Figure 6A). This "first
18 fragment" of the desired replacement sequence contains a
19 selectable marker sequence (most preferably the nptII gene)
20 at its end.

21 The DNA molecule also contains a "dual selection" gene
22 sequence which encodes a non-functional fragment of a gene
23 sequence for which both a positive and a negative selection
24 exists. An example of such a gene is the gpt gene when used
25 in the context of an hprt cell. Cells which express a
26 functional gpt gene can be selected for by their ability to
27 grow in HAT medium; Cells which lack a functional gpt gene
28 can be selected for by their ability to grow in the presence
29 of 6-thioguanine.

30 Homologous recombination results in the insertion of the
31 DNA molecule into the cell's genome at the region of
32 homology (Figure 6A). Importantly, since this step results
33 in the creation of a cell whose genome contains the

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1 selectable marker gene, it is possible to select for the
2 desired recombinational event.

3 In the second step of the method, a second DNA molecule
4 is provided to the cell. This second DNA molecule contains
5 a "second fragment" of the desired replacement sequence as
6 well as a sequence of the dual selection gene that, due to
7 an internal deletion, is incapable of encoding a functional
8 gene product. Homologous recombination results in the
9 insertion of the second DNA molecule into the cell's genome
10 in a manner so as to create a functional dual selection gene
11 (Figure 6B). Recombination also results in the integration
12 of a non-functional fragment of the dual selection gene.
13 Importantly, since this step results in the creation of a
14 cell whose genome contains a functional dual selection gene,
15 it is possible to select for the desired recombinational
16 event.

17 In the third step of the method, a third DNA molecule is
18 provided to the cell. This third DNA molecule contains both
19 the "first" and "second" fragments of the desired
20 replacement sequence. Homologous recombination results in
21 the insertion of the third DNA molecule into the cell's
22 genome in a manner so as to delete the functional dual
23 selection gene. The non-functional fragment of the dual
24 selection gene (formed in step 2) is not affected by the
25 recombination, and is retained (Figure 6C). Importantly,
26 since this step results in the creation of a cell whose
27 genome lacks the dual selection gene, it is possible to
28 select for the desired recombinational event.

29 In the fourth step of the method, a fourth DNA molecule
30 is provided to the cell. This fourth DNA molecule contains
31 a "third fragment" of the desired replacement sequence as
32 well as a sequence of the dual selection gene that, as in
33 step 2, is incapable of encoding a functional gene product

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1 due to an internal deletion. Homologous recombination
2 results in the insertion of the fourth DNA molecule into the
3 cell's genome in a manner so as to create a functional dual
4 selection gene (Figure 6D). Recombination also results in
5 the integration of a non-functional fragment of the dual
6 selection gene. Importantly, since this step results in the
7 creation of a cell whose genome contains a functional dual
8 selection gene, it is possible to select for the desired
9 recombinational event.

10 In the fifth step of the method, a fifth DNA molecule is
11 provided to the cell. This fifth DNA molecule contains both
12 the "second" and "third" fragments of the desired
13 replacement sequence. Homologous recombination results in
14 the insertion of the fifth DNA molecule into the cell's
15 genome in a manner so as to delete the functional dual
16 selection gene. The non-functional fragment of the dual
17 selection gene (formed in step 4) is not affected by the
18 recombination, and is retained (Figure 6C). Importantly,
19 since this step results in the creation of a cell whose
20 genome lacks the dual selection gene, it is possible to
21 select for the desired recombinational event.

22 As will be appreciated, the net effect of the above-
23 described steps is to produce a cell whose genome has been
24 engineered to contain a "first," "second," and "third"
25 "fragment" of a particular desired gene in a contiguous
26 manner. The steps may be repeated as desired in order to
27 introduce additional "fragments" into the cell's genome. In
28 this manner, cells can be constructed which contain
29 heterologous genes, chromosome fragments, or chromosomes,
30 that could not be introduced using a single vector. As
31 indicated above, each step of the method can be selected
32 for.

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1 In particular, this aspect of the present invention may
2 be used to produce "humanized" antibodies (i.e. non-human
3 antibodies which are non-immunogenic in a human) (Robinson,
4 R.R. et al., International Patent Publication
5 PCT/US86/02269; Akira, K. et al., European Patent
6 Application 184,187; Taniguchi, M., European Patent
7 Application 171,496; Morrison, S.L. et al., European Patent
8 Application 173,494; Neuberger, M.S. et al., PCT Application
9 WO 86/01533; Cabilly, S. et al., European Patent Application
10 125,023; Better, M. et al., Science 240:1041-1043 (1988);
11 Liu, A.Y. et al., Proc. Natl. Acad. Sci. USA 84:3439-3443
12 (1987); Liu, A.Y. et al., J. Immunol. 139:3521-3526 (1987);
13 Sun, L.K. et al., Proc. Natl. Acad. Sci. USA 84:214-218
14 (1987); Nishimura, Y. et al., Canc. Res. 47:999-1005 (1987);
15 Wood, C.R. et al., Nature 314:446-449 (1985)); Shaw et al.,
16 J. Natl. Cancer Inst. 80:1553-1559 (1988).

17 The method may also be used to produce animals having
18 superior resistance to disease, animals which constitute or
19 produce improved food sources, animals which provide fibers,
20 hides, etc. having more desirable characteristics. The
21 method may also be used to produce new animal models for
22 human genetic diseases. For example, the method may be used
23 to "humanize" the CD4 analog of an animal, and thus provide
24 an animal model for AIDS. Such animal models can be used
25 for drug testing, and thus hasten the development of new
26 therapies for genetic diseases.

27 In addition, the present invention permits the formation
28 of cells and of transgenic animals which contain mutations
29 in medically or clinically significant heterologous genes.
30 A gene is said to be medically or clinically significant if
31 it expresses an isotype of a protein associated with a human
32 or animal disease or condition. Examples of such genes
33 include the genes which encode: topoisomerase p180, 5- α

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1 reductase, ACAT, 5-lipoxygenase, the insulin receptor, the
2 interleukin-2 receptor, the epidermal growth factor
3 receptor, the serotonin receptor, the dopamine receptor, the
4 GABA receptor, the V₂ vasopressin receptors, G proteins
5 (signal transduction), phospholipase C proteins, and
6 insulin. A transgenic mouse produced by microinjection
7 which expresses human insulin was reported by Selden, R.F.
8 et al. (European Patent Publication No. 247,494, which
9 reference is incorporated herein by reference).

10 The transgenic cells and animals discussed above can be
11 used to study human gene regulation. For example,
12 transgenic animals which express a human isotype of
13 topoisomerase p180, 5- α reductase, ACAT, 5-lipoxygenase, or
14 hormone or cytokine receptors would have utility in in vivo
15 drug screening. The expression of topoisomerase p180 is
16 associated with resistance to chemotherapeutics. Thus,
17 agents which interfere with this enzyme could be used to
18 enhance the effectiveness of chemotherapy. An animal,
19 especially a rat, capable of expressing a human isotype of
20 5- α reductase (especially in the prostate gland) would be
21 highly desirable. ACAT is a key enzyme in lipid metabolism;
22 an animal model for its regulation would be extremely
23 valuable. Animals that express 5-lipoxygenase could be of
24 interest to many research programs, particularly to screen
25 isotype selective inhibitors. An animal which expressed
26 human hormone or cytokine receptor proteins would be
27 valuable in identifying agonists and antagonists of receptor
28 action. Similarly, an animal that expressed components of
29 the human signal transduction system (i.e. G proteins and
30 phospholipase Cs, etc.) could be used to study the
31 pathophysiologic consequences of disordered function of
32 these proteins.

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1 The present invention can be used to produce cells and
2 animals which express human isotypes of transport proteins
3 (i.e. proteins which facilitate or enable the transport of
4 other molecules or ions across membranes in the gut, blood
5 brain barrier, kidney, etc.). Such cells or animals can
6 then be used to study the role of such proteins in
7 metabolism. In particular, the extent and patterns of
8 conjugation mediated by such isotypes may be studied in
9 order to investigate the pharmacokinetic consequences of
10 specific differences in protein structure or sequence.
11 Glucoronide transferase, glycine conjugation and sulfation,
12 methylases, and glutathione conjugation are examples of
13 enzymes of particular interest in this regard.

14 The clearance of many compounds is mediated by
15 esterases. Cells or animals which express heterologous
16 isotypes of such esterases may be exploited in investigating
17 such clearance.

18 Cells or animals which express isotypes of proteins
19 involved in azo or nitro reduction would be desirable for
20 research on the processes of azo or nitro reduction.

21 Significantly, potential therapeutic agents are
22 frequently found to induce toxic effects in one animal model
23 but not in another animal model. To resolve the potential
24 of such agents, it is often necessary to determine the
25 metabolic patterns in various species, and to then determine
26 the toxicities of the metabolites. The present invention
27 permits one to produce transgenic cells or animals which
28 could facilitate such determinations.

29 The methods of the present invention may be used to
30 produce alterations in a regulatory region for a native gene
31 sequence. Thus, the invention provides a means for altering
32 the nature or control of transcription or translation of any
33 native gene sequence which is regulated by the regulatory

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1 region. For example, it is possible to introduce mutations
2 which remove feedback inhibition, and thus result in
3 increased gene expression. Similarly, it is possible to
4 impair the transcriptional capacity of a sequence in order
5 to decrease gene expression. Such alterations are
6 especially valuable in gene therapy protocols, and in the
7 development of improved animal models of human disease. For
8 example, the capacity to increase insulin gene transcription
9 or translation provides a potential genetic therapy for
10 diabetes. Similarly, the ability to impair the synthesis of
11 beta globin chains provides an animal model for beta-
12 thalassemia.

13 The methods of the present invention, quite apart from
14 their uses in veterinary and human medicine, may be used to
15 investigate gene regulation, expression and organization in
16 animals.

17 Since the methods of the present invention utilize
18 processes of DNA repair and recombination, agents which
19 inhibit or impair the present methods may act by affecting
20 these processes. Since agents which impair DNA repair and
21 recombination have potential antineoplastic utility, the
22 present invention provides a means for identifying novel
23 antineoplastic agents.

24 The present invention may additionally be used to
25 facilitate both the cloning of gene sequences, and the
26 mapping of chromosomes or chromosomal abnormalities.

27 Since the desired gene sequence need not be homologous
28 or analogous to any native gene sequence of the recipient
29 cell, the methods of the present invention permit one to
30 produce animals which contain and express foreign gene
31 sequences. If the cell expresses an analogous gene, the
32 desired gene sequence may be expressed in addition to such
33 analogous cellular genes (for example, an animal may express

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1 both a "humanized" receptor and an analogous native
2 receptor). Thus, for example, the invention provides a
3 means for producing animals which express important human
4 proteins (such as human interferons, tissue plasminogen
5 activator, hormones (such as insulin and growth hormone),
6 blood factors (such as Factor VIII), etc.).

7 In a second embodiment, the methods of the invention may
8 be used to introduce DNA into plant cells which can then be
9 manipulated in order to produce chimeric or transgenic
10 plants. The plants which may be produced through
11 application of the disclosed method include all
12 multicellular, higher (i.e. non-fungal) plants. A non-
13 fungal plant is any plant which is not a fungus or yeast.

14 In a third embodiment, the methods of the invention may
15 be used to introduce DNA into the somatic cells of an animal
16 (particularly mammals including humans) or plant in order to
17 provide a treatment for genetic disease (i.e. "gene
18 therapy"). The principles of gene therapy are disclosed by
19 Oldham, R.K. (In: Principles of Biotherapy, Raven Press, NY,
20 1987), and similar texts.

21 In this third embodiment, the genetic lesion which
22 causes the disease is replaced with a gene sequence encoding
23 a preferred gene product. Examples of such genetic lesions
24 are those responsible for diseases such as cystic fibrosis,
25 phenylketonuria, hemophilia, von Willebrand's Disease,
26 sickle cell anemia, thalassemia, galactosemia, fructose
27 intolerance, diseases of glycogen storage, hyper-
28 cholesterolemia, juvenile diabetes, hypothyroidism,
29 Alzheimer's Disease, Huntington's Disease, Gout, Lesch-Nyhan
30 Syndrome, etc. (Bondy, P.K. et al., In: Disorders of
31 Carbohydrate Metabolism, pp 221-340, Saunders (1974);
32 Coleman, J. et al., Molecular Mechanisms of Disease, Yale
33 University Press, (1975)). Disclosures of the methods and

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1 uses for gene therapy are provided by Boggs, S.S. (Int. J.
2 Cell Clon. 8:80-96 (1990)); Karson, E.M. (Biol. Reprod.
3 42:39-49 (1990)); Ledley, F.D., In: Biotechnology, A
4 Comprehensive Treatise, volume 7B, Gene Technology, VCH
5 Publishers, Inc. NY, pp 399-458 (1989)); all of which
6 references are incorporated herein by reference.

7 In a fourth embodiment, the methods of the invention may
8 be used to provide a treatment to protect recipient animals
9 or plants from exposure to viruses, insects or herbicides
10 (in the case of plants), insecticides, toxins, etc. In this
11 embodiment, the introduced gene would provide the recipient
12 with gene sequences capable of mediating either an enhanced
13 or novel expression of an enzyme, or other protein, capable
14 of, for example, degrading an herbicide or toxin. For
15 example, a plant cell may receive a gene sequence capable of
16 mediating an enhanced or novel expression of a chitinase,
17 thus conferring increased resistance to insect parasites.

18 When providing the desired gene sequence to the cells of
19 an animal, pharmaceutically acceptable carriers (i.e.
20 liposomes, etc.) are preferably employed. Such gene
21 sequences can be formulated according to known methods to
22 prepare pharmaceutically useful compositions, whereby these
23 materials, or their functional derivatives, are combined in
24 admixture with a pharmaceutically acceptable carrier
25 vehicle. Suitable vehicles and their formulation, are
26 described, for example, in Nicolau, C. et al. (Crit. Rev.
27 Ther. Drug Carrier Syst. 6:239-271 (1989)), which reference
28 is incorporated herein by reference.

29 In order to form a pharmaceutically acceptable
30 composition suitable for effective administration, such
31 compositions will contain an effective amount of the desired
32 gene sequence together with a suitable amount of carrier
33 vehicle.

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1 Additional pharmaceutical methods may be employed to
2 control the duration of action. Control release
3 preparations may be achieved through the use of polymers to
4 complex or absorb the desired gene sequence (either with or
5 without any associated carrier). The controlled delivery
6 may be exercised by selecting appropriate macromolecules
7 (for example polyesters, polyamino acids, polyvinyl,
8 pyrrolidone, ethylenevinylacetate, methylcellulose,
9 carboxymethylcellulose, or protamine, sulfate) and the
10 concentration of macromolecules as well as the methods of
11 incorporation in order to control release. Another possible
12 method to control the duration of action by controlled
13 release preparations is to incorporate the agent into
14 particles of a polymeric material such as polyesters,
15 polyamino acids, hydrogels, poly(lactic acid) or ethylene
16 vinylacetate copolymers. Alternatively, instead of
17 incorporating these agents into polymeric particles, it is
18 possible to entrap these materials in microcapsules
19 prepared, for example, by coacervation techniques or by
20 interfacial polymerization, for example, hydroxymethylcellu-
21 lose or gelatine-microcapsules and poly(methylmethacrylate)
22 microcapsules, respectively, or in colloidal drug delivery
23 systems, for example, liposomes, albumin microspheres,
24 microemulsions, nanoparticles, and nanocapsules or in
25 macroemulsions.

26 In a fifth embodiment, the methods of the present
27 invention may be used to improve the food or fiber
28 characteristics of plants or non-human animals. For
29 example, the methods can be used to increase the overall
30 levels of protein synthesis thereby resulting in faster
31 growing plants or non-human animals, or in the production of
32 plants and non-human animals which have increased food
33 value.

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1 Having now generally described the invention, the same
2 will be more readily understood through reference to the
3 following examples which are provided by way of
4 illustration, and are not intended to be limiting of th
5 present invention, unless specified.

6
7 EXAMPLE 1
8 ELECTROPORATION

9
10 Electroporation was performed as follows:

11
12 DNA Preparation:

13
14 DNA used for electroporation was purified by CsCl
15 gradient centrifugation. A large-scale digest of this
16 purified DNA was prepared by incubating the DNA with an
17 appropriate restriction enzyme. The large-scale digest was
18 examined for complete digestion by running 500 ng on a
19 minigel. The DNA concentration of the large-scale digest
20 should be no higher than 1 $\mu\text{g}/\mu\text{l}$.

21 The large-scale digest was then extracted once with an
22 equal volume of phenol/chloroform and once with an equal
23 volume of chloroform. The DNA was precipitated with 2.4
24 volumes of ethanol, pelleted by centrifugation, and dried
25 using a Speed-Vac.

26 The pelleted DNA was then resuspended at the desired
27 concentration (usually 1 $\mu\text{g}/\mu\text{l}$) in a sterile Tris-EDTA
28 buffer such as 0.1X TE (25 μl of DNA per electroporation).
29 The concentration of the DNA was then measured with a
30 fluorometer.

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Preparation of Cells for Electroporation:

Embryonic stem cells of the AB1 cell line were cultured to approximately 80% confluence according to the methods of E.J. Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E.J. Robertson, Ed.), IRL Press, Oxford, 1987, pp 71-112). Cells were cultured in the presence of stomal cells which expressed lif into the culture medium. Cells were passaged 1:2 the day before electroporation, and fed 4 hours before harvesting.

Cells were harvested by trypsinizing the cells, and by resuspending in media (cells from 2 x 10 cm plates were combined in a total volume of 10 ml in a 15 ml tube).

The cells were pelleted by centrifugation, and the supernatant was removed by aspiration. The cells were then resuspended in 10 ml of phosphate buffered saline and the total number of cells was determined by counting a 20 μ l aliquot. The usual yield is 30×10^6 cells per 10 cm plate.

The cells were then pelleted by centrifugation and the supernatant was removed by aspiration. Cells were resuspended at a density of 11×10^6 cells/ml. A 20 μ l aliquot was counted to confirm this cell density.

Electroporation

Cells, prepared as described above, were incubated in the presence of an appropriate amount of DNA in a 15 ml tube. 25 μ l of DNA and 0.9 ml of cells were used for each electroporation.

The mixture was allowed to incubate at room temperature for 5 minutes (this step may, however, be omitted).

The cell/DNA mixture was then carefully aliquoted into electroporation cuvettes (0.9 ml per cuvette; the volume is

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1 important). The cuvette was placed in the electroporation
2 holder with the foil electrodes in contact with the metal
3 holding clips.

4 Electroporation was accomplished using a Biorad
5 GenePulser set at 230V, 500 μ F (this requires a capacitance
6 extender). The time constant should read between 5.6 and
7 7.0.

8 The cuvette was left at room temperature for 5 minutes
9 and then the cells were plated at an appropriate density (up
10 to 2×10^7 cells/100 mm plate or 6×10^6 cells/60 mm plate).
11 When G418 was used as a selective agent, this cell density
12 should not be exceeded since G418 takes 3-4 days before
13 killing starts and plates will become over-confluent. When
14 G418 selection was to be applied, it is applied 24 hours
15 post-electroporation. G418 concentration must be titrated
16 for every batch.

17 The plate(s) were re-fed with fresh media + G418 every
18 day for the first 6-7 days (until colonies are visible and
19 most cell debris has been removed). If using FIAU (0.2 μ M)
20 selection, this may proceed simultaneously.

21 The typical yield for RV4.0 (Thomas, K.R. et al., Cell
22 51:503-512 (1987)) is up to 10^4 colonies/ 10^7 cells/100 mm
23 plate. Although this yield may be significantly (and
24 unpredictably) different from the yield obtained when other
25 constructs are used, the use of the method always results in
26 the recovery of some colonies of cells which contain the
27 electroporated DNA.

28 Colonies may be picked as early as 8 days. It is most
29 preferred to pick colonies at around 10-11 days. Colonies
30 may, however, be recovered up to 18-21 days after the
31 electroporation.
32

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EXAMPLE 2
CO-ELECTROPORATION OF ES CELLS

To illustrate the invention, embryonic stem ("ES") cells were co-electroporated with a 4.5 kb nptII-containing vector (pPGKneobpA) which had been linearized by treatment with XhoI restriction endonuclease, and with the 6.5 kb HPRT vector, AD 8 (linearized with SacI) (Figure 7). Electroporation (230 V, 500 μ F) were done on 0.9 ml aliquot of CCEp24 cells (7.5×10^6 cells/ml).

The electroporation reactions were conducted using molar ratios of 1:1, 1:10, and 1:100 (nptII DNA:HPRT DNA). The total amount of DNA provided was either 25, 50, 100, or 200 μ g. The vectors used in this experiment are illustrated in Figure 7. The results of this experiment are shown in Table 1.

TABLE 1
CO-ELECTROPORATION OF nptII AND hprt GENE SEQUENCES

Average of Number of Colonies Formed per 1×10^6 Cells
(μ g of DNA (# = Number of trials averaged))

Ratio of DNA	200			100			50			25		
	G418 ^R	TG ^R	#	G418 ^R	TG ^R	#	G418 ^R	TG ^R	#	G418 ^R	TG ^R	#
1:1	233	2.7	3	101	1.5	3	64	0	3	23	0	5
1:10	46	0	3	16	0	5	8.7	0	7	nd	nd	
1:100	8	0.2	5	4.3	0	7	1.6	0	7	nd	nd	

This experiment shows that co-electroporation of an hprt gene sequence with an nptII-containing gene sequence in the presence of selection for only the nptII-containing sequence, resulted in recombination of both the nptII and hprt DNA molecules.

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The frequencies of recombination are shown in Table 2 below.

TABLE 2
FREQUENCY OF RECOMBINATION

Expt	Ratio Neo:Hprt	[DNA] μg/ml	G418 ^R /10 ⁵	TG ^R /10 ⁷	TG ^R /G418 ^R
A	1:1	200	23.3	2.6	1/873
B	1:1	100	10.1	1.0	1/1010
C	1:100	200	0.8	0.2	1/400*
Cont	---	25	10.8	2.7	1/402

The reactions were carried out as described above. The reproducibility of the experimental results was examined. The results of this experiment are shown in Table 3.

TABLE 3
EFFECT OF MODIFIED CO-ELECTROPORATION PROTOCOL ON
RECOMBINATION FREQUENCY

Molar ratios	DNA per zap (μg)	# of zap	G418 ^R /HPRT- colonies (total)	HPRT- G418R	HPRT- (per cell transfected) (X 10 ⁻⁹)	G418 ^R (X 10 ⁻⁶)
Neo:						
1:1	200	8	16,150 / 32	1/504	400	202
	100	3	3,030 / 3	1/1,010	100	105
	50	3	1,920 / 0		67	
	25	5	1,150 / 0		24	
1:10	200	16	608 / 7	1/868	43	47
	100	5	800 / 0		17	
	50	7	609 / 0		9	
1:100	200	5	400 / 1	1/400	8	
	100	7	300 / 0		4.5	
	50	7	112 / 0		1.7	

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EXAMPLE 3
HOMOLOGOUS RECOMBINATION

In order to investigate the chromosomal structure which is produced by the recombination of the vectors of the above-described vectors into the chromosomes of recipient cells, the following experiments were conducted.

For this purpose, a vector was used which contained a 6.5 kb region of homology with the cellular hprt locus. The vector also contained the np^tII gene, as a selectable maker. The vector was linearized with XhoI and provided to ES cells by electroporation, as described above. Cells which became resistant to G418 were selected and their DNA was analyzed to determine if it contained restriction fragments that were consistent with the predicted integration structure.

The vector used, and the predicted integration structure are illustrated in Figure 8. Gel electrophoresis of restriction digests of cellular DNA confirmed that the G418 resistant cells contained the hprt structure shown in Figure 8. This finding confirmed that the vector had integrated into the chromosome of the cell by homologous recombination at the hprt locus.

EXAMPLE 4
REVERSION OF RECOMBINANTS

The effect of the size of the region of homology carried by the vector on the reversion frequency of recombinants was determined. Recombinants containing a vector having 6.8 kb of homology with the hprt locus were prepared as described in Example 3. Using the same method, recombinants were also prepared which contained a similar vector having only 1.3 kb

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of homology with the hprt locus. The structures of the insertion site of the 6.8 kb vector is illustrated in Figure 8. The reversion frequency of the two constructs is shown in Table 4. The structure obtained from the reversion of the insertion is shown in Figure 9.

TABLE 4
REVERSION FREQUENCY

<u>Duplication</u>	<u># Clones</u>	<u># Revertible</u>	<u>Frequency x10⁻⁵</u>
6.8 kb	19	19	3.3 to 0.2
1.3 kb	2	2	1.2 to 0.3

EXAMPLE 5
TARGETING FREQUENCY OF INSERTION AND REPLACEMENT VECTORS

A series of different vectors were used to investigate the targeting frequency achieved through the use of the methods of the invention. These vectors contained 6.8 kb of homology with the murine hprt gene and had regions of heterology either at the linearization site or internally (Figure 2).

For this purpose, 10^8 cells were electroporated into ES cells, prepared as described above, and plated onto 10 x 90 mm plates. After 24 hours G418 (at 350 μ g/ml) was added to the media. After 5 days selection 10^{-5} M 6-thioguanine was added to 9 plates, 1 was retained under G418 selection as the transfection control. Selection was continued for an additional 7 days. Colonies were scored at this time and expanded for southern analysis as separate clones. Targeting efficiencies are detailed for each of the vectors (Figure 2; Table 5).

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Southern analysis showed that the majority of the 6-TG^R clones had the predicted integration structure depicted for HindIII digestion in Figure 8.

Reversion of the hp^rt clones was done by measuring HAT^R. Cells were clonally expanded under 6-TG selection to prevent "jackpot" effects caused by the early recombinational loss of the duplicated element giving rise to a large number of colonies by cell division. When 10⁷ cells were obtained, the cells were reseeded onto 90 mm plates without selection for 48 hours. After 48 hours HAT selection was applied and resistant colonies were scored 10 days later, typically 20 to 200 colonies were observed per 10⁷ cells plated (Table 4). Every clone examined reverted at a similar frequency.

TABLE 5
REPLACEMENT AND INSERTION VECTORS: TARGETING AND FREQUENCY

<u>Gene</u>	<u>Homology</u>	<u>Vector</u>	<u>Frequency</u>	
Hprt	6.8 kb	RV	1/300	10X
Hprt	6.8 kb	IV	1/32	
Hprt	1.3 kb	RV	<1/5000	12x
Hprt	minimum 6.8 kb	IV	1/400	
Hox2.6	3.2 kb	IV+	1/33	

RV=Replacement Vector; IV=Insertion Vector

EXAMPLE 6 SELECTION FOR HOMOLOGOUS RECOMBINATION

It is possible to use "Poly A Selection" in order to enhance the selection of cells which have integrated the introduced DNA by homologous recombination.

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1 If an introduced DNA molecule were to integrate at
2 random into the host chromosome, it would generally not
3 integrate at a site adjacent to a necessary 3'
4 polyadenylation site. Thus, the mRNA produced by the
5 transcription of such randomly inserted constructs would
6 generally lack polyadenylation. This fact can be exploited
7 by using vectors which permit one to select for a
8 recombinational event that results in integration adjacent
9 to the natural polyadenylation site of the introduced gene
10 sequence (i.e. by homologous recombination rather than by
11 random insertion).

12 To illustrate this aspect of the invention, three
13 vectors were constructed which contain fragments of the hprt
14 gene (Figure 10). As shown in Figure 10, the vectors
15 contain exons 7, 8, and 9 of the hprt gene. The
16 polyadenylation site is located in exon 9. A HindIII site
17 is present within exon 9, and an EcoRI site is located after
18 the end of the exon.

19 The first vector employed contained a 5.0 kb region, and
20 thus contained the polyadenylation site of exon 9 (Vector 6,
21 Figure 10). As shown in Table 6, the frequency of insertion
22 was high (i.e. frequency of G418 resistant colonies was 24×10^{-5}),
23 but only 1/941 colonies showed the dual thioguanine
24 resistance and G418 resistance which would characterize a
25 desired recombinant (i.e. a recombinant in which integration
26 had resulted in an intact hprt gene and an intact nptII
27 gene). Thus, some random integration is occurring.

28 Similarly, when a vector of 3.5 kb was employed (Vector
29 10) which contained DNA from the XbaI site to the EcoRI site
30 of Vector 6, the frequency of insertion was high (i.e.
31 frequency of G418 resistant colonies was 21×10^{-5}), but only
32 1/770 colonies showed the dual thioguanine resistance and
33 G418 resistance which would characterize a desired

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recombinant (Table 6). This finding demonstrates that some random integration is occurring.

If, however, a vector is employed which lacks the polyadenylation site of exon 9 (i.e. Vector 9), random integration does not result in expression of a functional nptII transcript. Thus, the frequency of G418 resistant colonies is low (1.4×10^{-5}). Since the number of colonies evidencing random integration is suppressed, the overall frequency of recovery of the desired recombinants is enhanced (i.e. an overall efficiency of 1/100 for the dual resistant colonies (Table 6). Thus, the poly A selection results in an approximate increase of overall efficiency of nearly 10 fold. Poly A selection may therefore be advantageously used in situations where one desires to minimize or avoid the screening of colonies to identify random versus homologous recombinants.

TABLE 6
POLY A SELECTION

VECTOR #	SIZE (kb)	G418 ^R ($\times 10^{-5}$)	TG ^R ($\times 10^{-7}$)	TG ^R /G418 ^R
6	5.0	24	2.5	1/941
9	3.0	1.4	1.4	1/100
10	3.5	21	2.7	1/770

EXAMPLE 7
INTRODUCTION OF SUBTLE MUTATIONS IN THE C-SRC LOCUS

The methods of the present invention were further illustrated by their use to produce cells having precise and subtle mutations in the c-src locus of ES cells. The c-src locus contains several exons, which are designated as "boxed" regions 2 and 3' in Figure 11. As shown in Figure

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11A, the natural allele of exon 3' does not contain a HindIII site.

The sequence of a portion of exon 3' is shown in Figure 11C. As shown in Figure 11C, a 9 bp insertion into this exon will result in the formation of a HindIII site.

To accomplish this change in the sequence of exon 3', a vector (src 14) was prepared. As shown in Figure 11B, the src 14 vector is homologous to a region of the c-src locus. The exon 3' sequence of the vector, however, has been altered to contain the 9 base pair insertion needed to create a HindIII site (Figure 11C).

The src 14 vector was introduced into ES cells by co-electroporation with a second vector (PGKneo) that contained the nptII gene, at a total DNA concentration of 25 µg/ml and a molar ratio of 1:5 (neo vector to targeting vector) in the manner described above.

Cells were cultured in the presence of G418 for 12 days in order to select for recombinant cells in which the nptII gene had integrated. These recombinant cells were then screened, using PCR, for cells which had undergone a recombinational event resulting in the replacement of the natural exon 3' locus with the HindIII site-containing exon 3' sequence of the src 14 vector.

Southern analysis of the colonies identified by PCR screening using probes B and C (Figure 11B) demonstrated that the natural exon 3' locus had been altered, as desired, to contain a HindIII site. This experiment demonstrated that subtle insertions can be introduced into any cellular gene.

To further illustrate the capacity of the present invention to introduce complex, predetermined mutations into the genome of a recipient cell, exon 3" of the c-src gene of

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1 an ES cell was mutated to contain two different substitution
2 mutations.

3 As shown in Figure 12A, the natural allele of exon 3"
4 does not contain either an NheI site or an EcoRI site. As
5 shown in Figure 12C, however, the replacement of the natural
6 sequence ACC TGG TTC of exon 3" with the sequence TAG CTA
7 GCT will result in the formation of an NheI site.
8 Similarly, replacement of ACA with GAA in exon 3" will
9 create an EcoRI site (Figure 12C).

10 To accomplish these changes in the sequence of exon ",
11 a vector (src 33) was prepared. As shown in Figure 12B, the
12 src 33 vector is homologous to a region of the c-src locus.
13 The exon 3" sequence of the vector, however, has been
14 altered to contain the substitutions indicated above (Figure
15 12C).

16 The src 33 vector was introduced into ES cells by
17 electroporation, in concert with a second vector that
18 contained the nptII gene, in the manner described above.
19 Cells were cultured in the presence of G418 in order to
20 select for recombinant cells in which the nptII gene had
21 integrated. These recombinant cells were then screened,
22 using PCR, for cells which had undergone a second
23 recombinational event resulting in the replacement of the
24 natural exon 3" locus with the exon 3" sequence of the src
25 33 vector.

26 Southern analysis of the colonies identified by PCR
27 screening using probes A and C (Figure 12C) demonstrated
28 that the natural exon 3" locus had been altered, as desired,
29 to contain both the NheI and the EcoRI sites. This
30 experiment demonstrated that subtle substitutions can be
31 introduced into any cellular gene.

32 While the invention has been described in connection
33 with specific embodiments thereof, it will be understood

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1 that it is capable of further modifications and this
2 application is intended to cover any variations, uses, or
3 adaptations of the invention following, in general, the
4 principles of the invention and including such departures
5 from the present disclosure as come within known or
6 customary practice within the art to which the invention
7 pertains and as may be applied to the essential features
8 hereinbefore set forth and as follows in the scope of the
9 appended claims.

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1
2 WHAT IS CLAIMED IS:
3

4 1. A method for obtaining a desired animal or non-fungal
5 plant cell which contains a desired non-selectable gene
6 sequence inserted within a predetermined gene sequence of
7 said cell's genome, which method comprises:

8 A. incubating a precursor cell with a DNA molecule
9 containing said desired non-selectable gene sequence,
10 wherein said DNA molecule additionally contains two regions
11 of homology which flank said desired gene sequence, and
12 which are sufficient to permit said desired gene sequence to
13 undergo homologous recombination with said predetermined
14 gene sequence of said genome of said precursor cell;

15 B. causing said DNA molecule to be introduced into
16 said precursor cell;

17 C. permitting said introduced DNA molecule to
18 undergo homologous recombination with said predetermined
19 gene sequence of said genome of said precursor cell to
20 thereby produce said desired cell wherein said desired non-
21 selectable gene sequence has been inserted into said
22 predetermined gene sequence; and

23 D. recovering said desired cell.
24

25 2. The method of claim 1 wherein said DNA molecule
26 contains a detectable marker gene sequence.
27

28 3. The method of claim 1 wherein said DNA molecule is
29 introduced into said precursor cell by subjecting said
30 precursor cell and said DNA molecule to electroporation.
31

32 4. The method of claim 3 wherein in step B, said
33 precursor cell is simultaneously subjected to

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1 electroporation with a second DNA molecule, said second DNA
2 molecule containing a detectable marker gene sequence.

3
4 5. The method of claim 1 wherein said desired cell is a
5 non-fungal plant cell.

6
7 6. The method of claim 1 wherein said desired cell is an
8 animal cell.

9
10 7. The method of claim 6 wherein said animal cell is a
11 somatic cell.

12
13 8. The method of claim 7 wherein said animal cell is of
14 an animal selected from the group consisting of a chicken,
15 a mouse, a rat, a hamster, a rabbit, a sheep, a goat, a
16 fish, a pig, a cow or bull, a non-human primate and a human.

17
18 9. The method of claim 6 wherein said animal cell is a
19 pluripotent cell.

20
21 10. The method of claim 9 wherein said animal cell is of
22 an animal selected from the group consisting of a chicken,
23 a mouse, a rat, a hamster, a rabbit, a sheep, a goat, a
24 fish, a pig, a cow or bull, and a non-human primate.

25
26 11. The method of claim 9 wherein said pluripotent cell
27 is an embryonic stem cell.
28

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12. The method of any one of claims 1-3 wherein said desired gene sequence is substantially homologous to said predetermined gene sequence of said precursor cell.

13. The method of claim 12 wherein said desired gene sequence is an analog of said predetermined sequence of said precursor cell.

14. The method of claim 12 wherein said desired gene sequence is a human analog of said predetermined sequence of said precursor cell.

15. The method of claim 12 wherein said desired cell is a non-human cell which expresses said desired gene sequence.

16. The method of claim 12 wherein said desired gene sequence encodes a protein selected from the group consisting of: a hormone, an immunoglobulin, a receptor molecule, a ligand of a receptor molecule, and an enzyme.

17. A non-fungal plant cell which contains an introduced recombinant DNA molecule containing a desired gene sequence, said desired gene sequence being flanked by regions of homology which are sufficient to permit said desired gene sequence to undergo homologous recombination with a predetermined gene sequence of the genome of said cell.

18. A non-human animal cell which contains an introduced recombinant DNA molecule containing a desired gene sequence, said desired gene sequence being flanked by regions of homology which are sufficient to permit said desired gene sequence to undergo homologous recombination with a predetermined gene sequence of the genome of said cell.

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1
2 19. The desired cell produced by the methods of any one
3 of claims 1-3.

4
5 20. The desired cell produced by the method of claim 11.
6

7 21. The desired cell produced by the method of claim 12.
8

9 22. A non-human animal containing a cell derived from the
10 desired cell of claim 19, wherein said animal is either a
11 chimeric or a transgenic animal.
12

13 23. The non-human animal of claim 22, wherein said animal
14 and said desired cell are of the same species, and wherein
15 said species is selected from the group consisting of: a
16 chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a
17 goat, a fish, a pig, a cow or bull, and a non-human primate.
18

19 24. A non-human animal containing a cell derived from the
20 desired cell of claim 20, wherein said animal is either a
21 chimeric or a transgenic animal.
22

23 25. The non-human animal of claim 24, wherein said animal
24 and said desired cell are of the same species, and wherein
25 said species is selected from the group consisting of: a
26 chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a
27 goat, a fish, a pig, a cow or bull, and a non-human primate.
28

29 26. A non-human animal containing a cell derived from the
30 desired cell of claim 21, or a descendant thereof, wherein
31 said animal is either a chimeric or a transgenic animal.
32

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1 27. The non-human animal of claim 26, wherein said animal
2 and said desired cell are of the same species, and wherein
3 said species is selected from the group consisting of: a
4 chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a
5 goat, a fish, a pig, a cow or bull, and a non-human primate.
6

7 28. A non-fungal plant containing a cell derived from the
8 desired cell of claim 5, or a descendant thereof, wherein
9 said non-fungal plant is either a chimeric or a transgenic
10 plant.
11

12 29. A method of gene therapy which comprises introducing
13 to a recipient in need of such therapy, a desired non-
14 selectable gene sequence, said method comprising:
15

16 A. providing to said recipient an effective amount
17 of a DNA molecule containing said desired non-selectable
18 gene sequence, wherein said DNA molecule additionally
19 contains two regions of homology which flank said desired
20 gene sequence, and which are sufficient to permit said
21 desired gene sequence to undergo homologous recombination
22 with a predetermined gene sequence present in a precursor
23 cell of said recipient;
24

25 B. permitting said DNA molecule to be introduced
26 into said precursor cell;
27

28 C. permitting said introduced DNA molecule to
29 undergo homologous recombination with said predetermined
30 gene sequence of said genome of said precursor cell to
31 thereby produce a desired cell wherein said desired non-
32 selectable gene sequence has been inserted into said
33 predetermined gene sequence; and wherein the presence or
expression of said introduced gene sequence in said cell of
said recipient comprises said gene therapy.

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1 30. The method of claim 29 wherein said recipient is a
2 non-fungal plant.

3
4 31. The method of claim 29 wherein said recipient is an
5 animal.

6
7 32. The method of claim 31 wherein said animal is
8 selected from the group consisting of: a chicken, a mouse,
9 a rat, a hamster, a rabbit, a sheep, a goat, a fish, a pig,
10 a cow or bull, a non-human primate and a human.

11
12 33. The method of claim 32, wherein said animal is a
13 human.

14
15 34. A method for obtaining a desired animal or non-fungal
16 plant cell which contains a desired non-selectable gene
17 sequence inserted within a predetermined gene sequence of
18 said cell's genome, which method comprises:

19 A. incubating a precursor cell under non-selective
20 culture conditions, or under a first set of selective
21 culture conditions, with a DNA molecule containing:

22 i) said desired non-selectable gene sequence,
23 wherein said DNA molecule additionally contains
24 two regions of homology which flank said desired
25 gene sequence, and which are sufficient to permit
26 said desired gene sequence to undergo homologous
27 recombination with said predetermined gene
28 sequence of said genome of said precursor cell;
29 and

30 ii) a selectable gene sequence whose presence or
31 expression in said precursor cell can be selected
32 for by culturing said cell under said first set
33 of selective culture conditions, and whose

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1 presence or expression in said precursor cell can
2 be selected against by culturing said cell under
3 a second set of selective culture conditions;

4 B. permitting said DNA molecule to be introduced
5 into said precursor cell;

6 C. permitting said introduced DNA molecule to
7 undergo homologous recombination with said predetermined
8 gene sequence of said genome of said precursor cell to
9 thereby produce said desired cell wherein said desired non-
10 selectable gene sequence has been inserted into said
11 predetermined gene sequence; and

12 D. recovering said desired cell by culturing said
13 cell under said first set of selective culture conditions,
14 by then permitting said cell to undergo intrachromosomal
15 recombination under non-selective culture conditions, and by
16 then incubating said cell under said second set of selective
17 culture conditions.
18

19 35. The method of claim 34, wherein said cell is
20 deficient in HPRT enzyme, and wherein said selectable gene
21 sequence expresses an active HPRT enzyme, and wherein said
22 first set of selective culture conditions comprises
23 incubation of said cell under conditions in which the
24 presence of an active HPRT enzyme in said cell is required
25 for growth, and wherein said second set of selective culture
26 conditions comprises incubation of said cell under
27 conditions in which the absence of an active HPRT enzyme in
28 said cell is required for growth.
29

30 36. The method of claim 34, wherein said cell is
31 deficient in APRT enzyme, and wherein said selectable gene
32 sequence expresses an active APRT enzyme, and wherein said
33 first set of selective culture conditions comprises

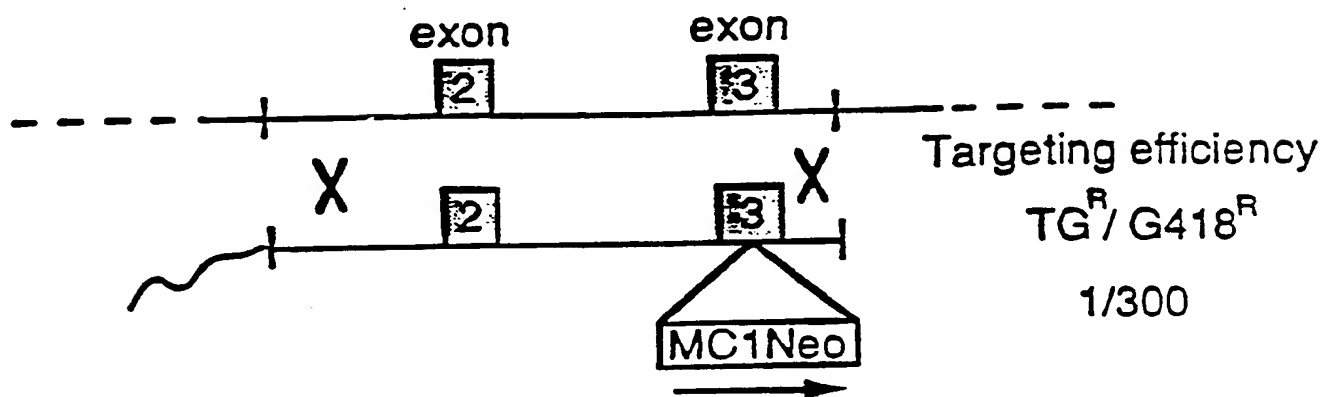
-79-

1 incubation of said cell under conditions in which the
2 presence of an active APRT enzyme in said cell is required
3 for growth, and wherein said second set of selective culture
4 conditions comprises incubation of said cell under
5 conditions in which the absence of an active APRT enzyme in
6 said cell is required for growth.
7

8 37. The method of claim 34, wherein said cell is
9 deficient in TK enzyme, and wherein said selectable gene
10 sequence expresses an active TK enzyme, and wherein said
11 first set of selective culture conditions comprises
12 incubation of said cell under conditions in which the
13 presence of an active TK enzyme in said cell is required for
14 growth, and wherein said second set of selective culture
15 conditions comprises incubation of said cell under
16 conditions in which the absence of an active TK enzyme in
17 said cell is required for growth.

- 1 / 17

1A Replacement vectors: 6.8kb Homology which target the hprt locus



1B Insertion Vectors : 6.8kb homology

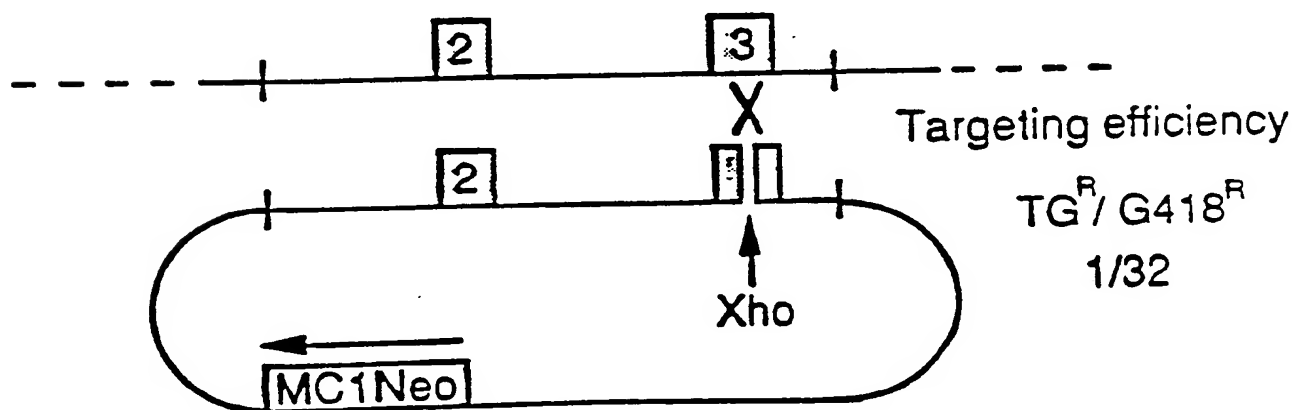
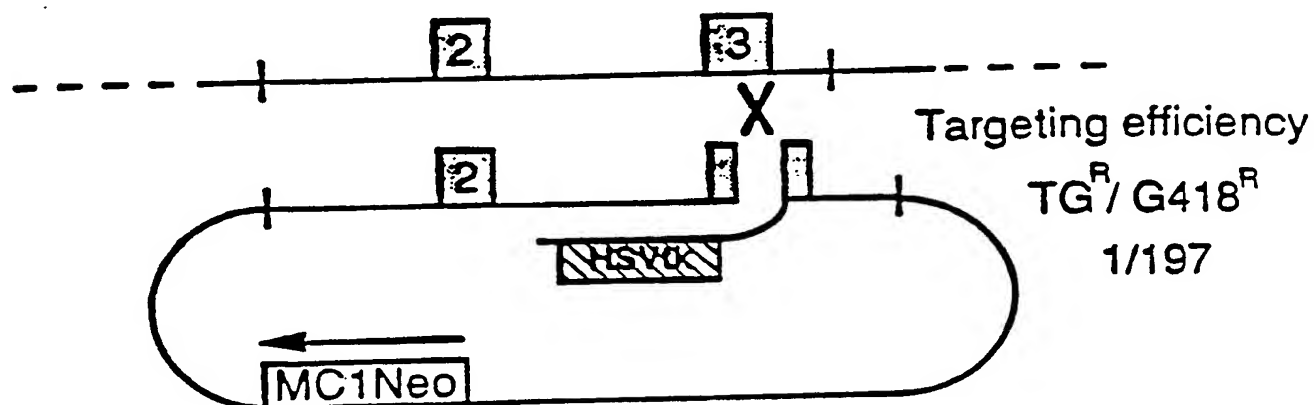


FIGURE 1

- 2 / 17

A. Insertion vectors with heterology at the insertion site: 2kb
insertion at the linearization site



2B. Insertion vectors with heterology at the insertion site: 26bp
insertion at the linearization site

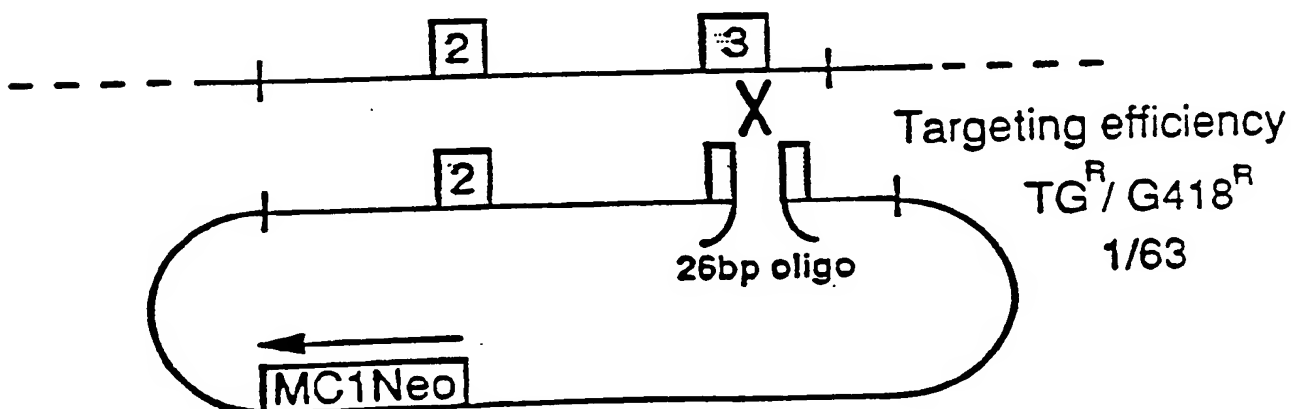


FIGURE 2

- 3 / 17

2c Insertion vectors with added heterology: Internal to the homology

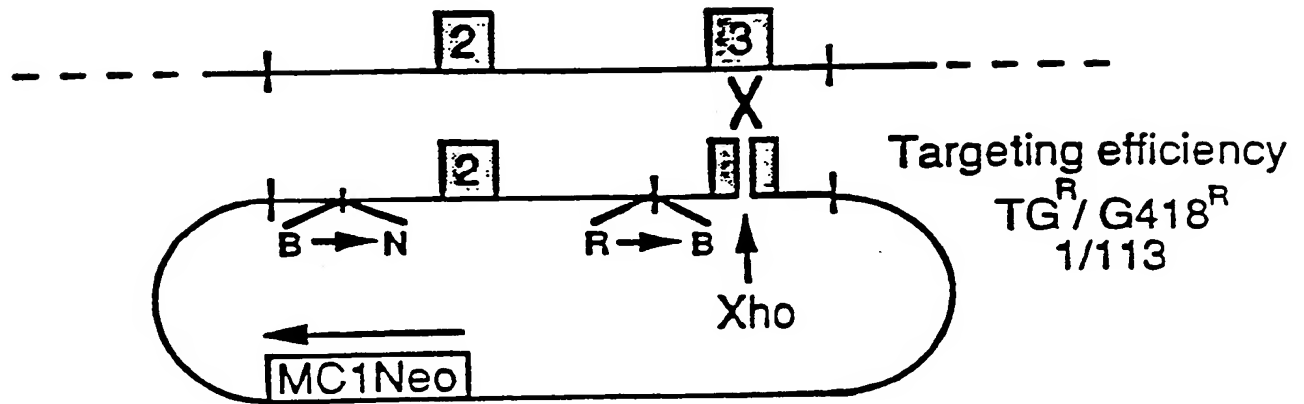
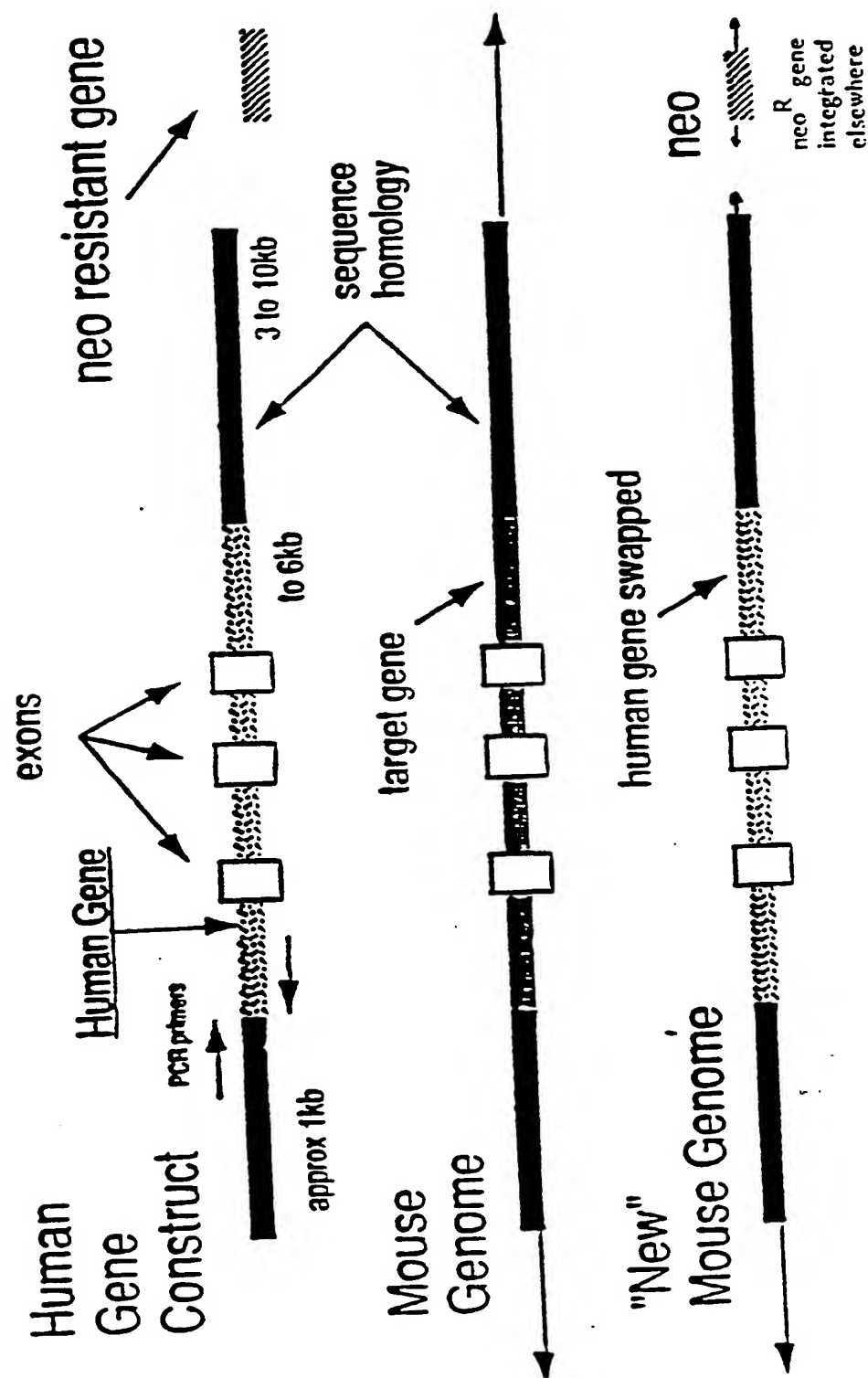


FIGURE 2 (continued)

- 4 / 17

FIGURE 3

Genetic Replacement Technology



- 5 / 17

^ Step#1, homologous recombination: Adding the human replacement with an insertion vector.

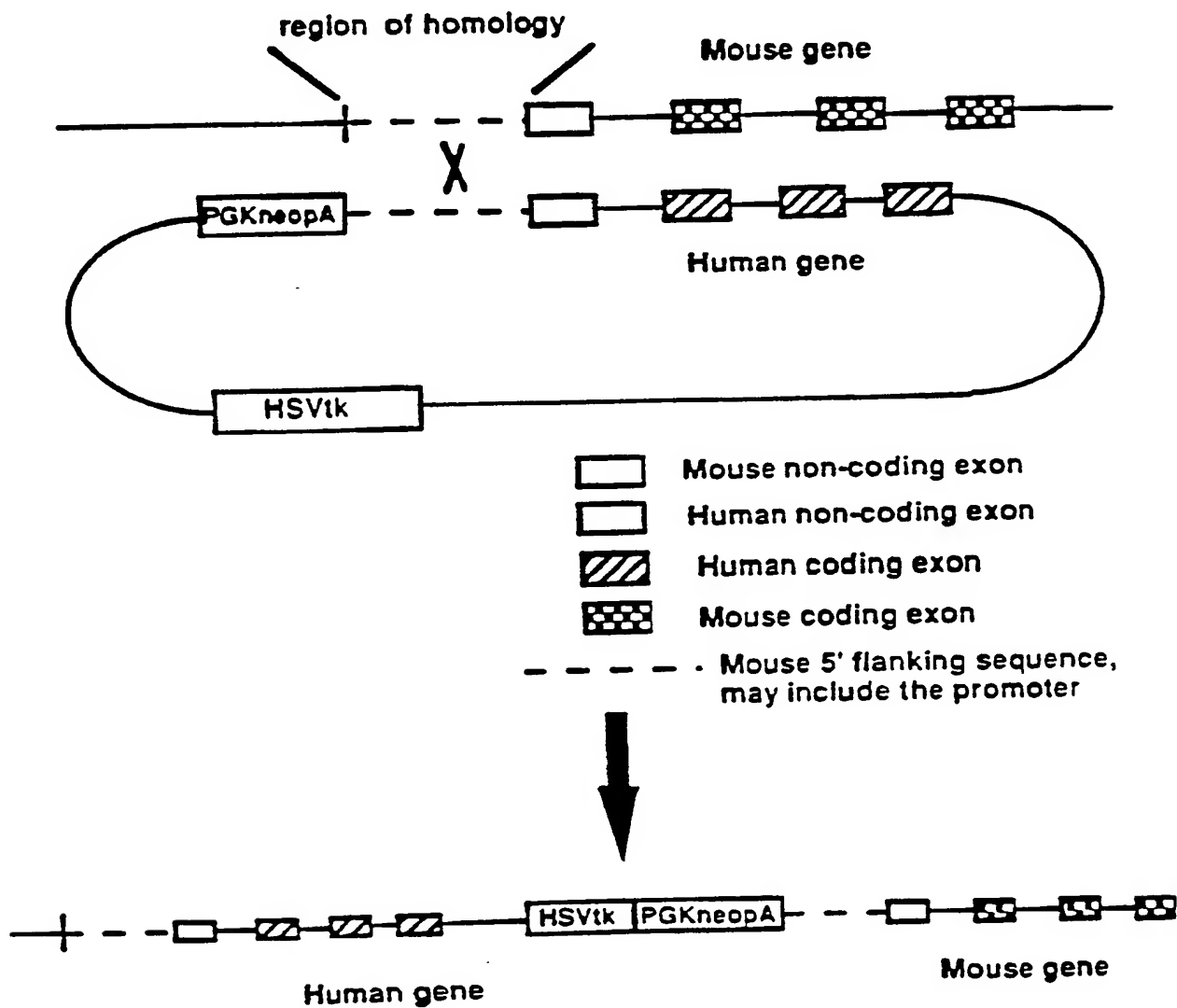


FIGURE 4

- 6 / 17

Step#2: Reconstruct junction, remove duplicated promoter, add additional 3' human sequences. Select in - FIAU (100%)

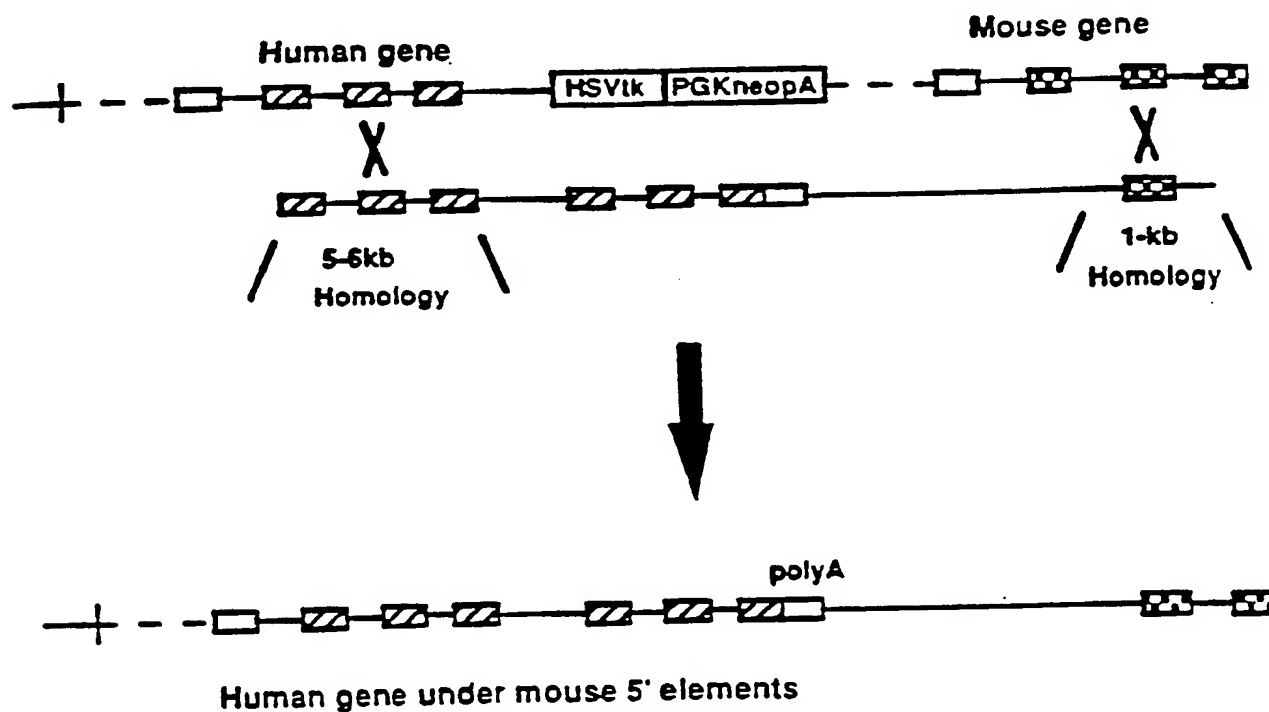
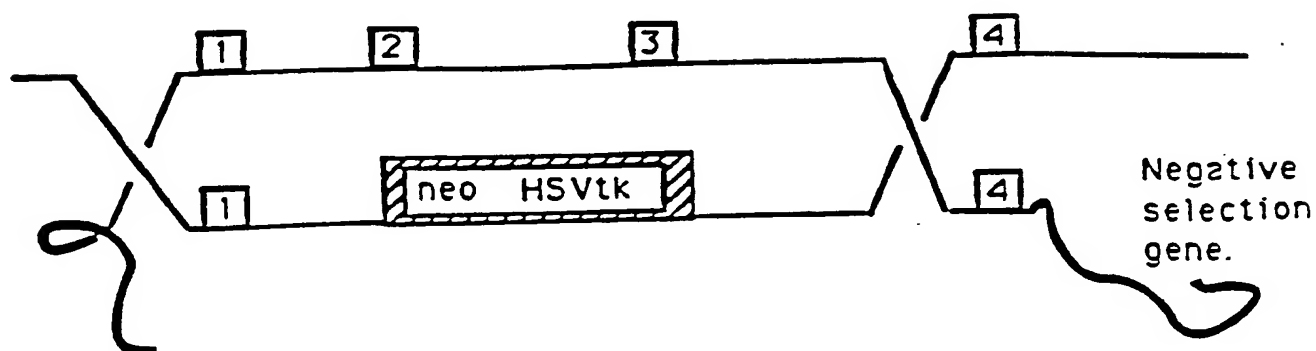


FIGURE 4 (continued)

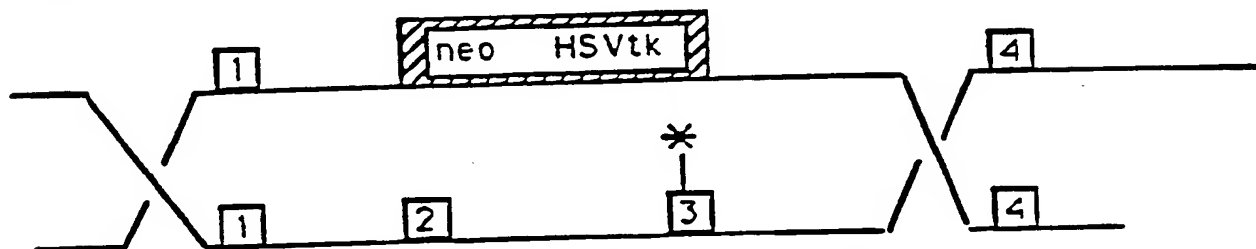
- 7 / 17

Selective introduction of small and large genomic changes

Step 1 - Standard Replacement vector



Step 2 A - Introduction of small mutations



Step 2 B complete or partial replacement with homologous or heterologous sequences.

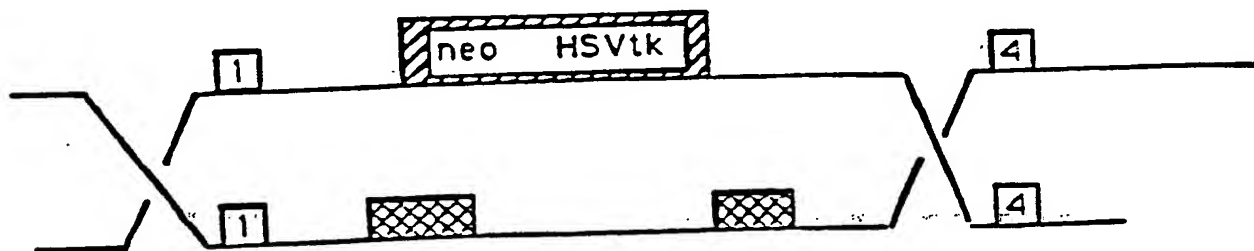
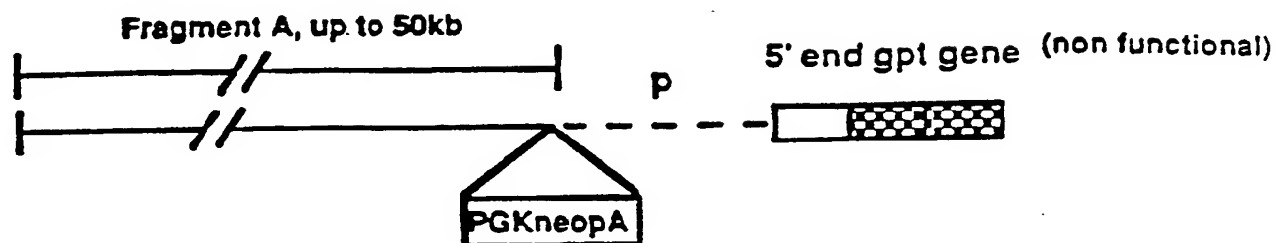


FIGURE 5

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6A

Step #1 Set up the initial target

6B

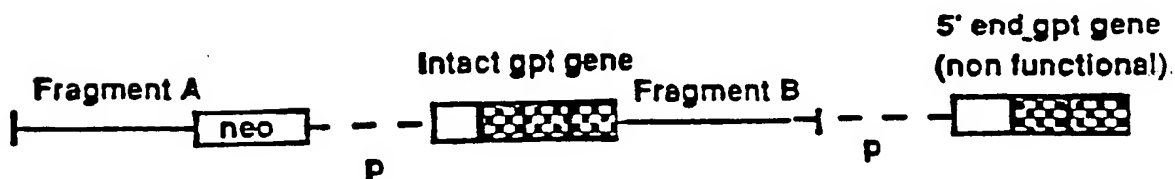
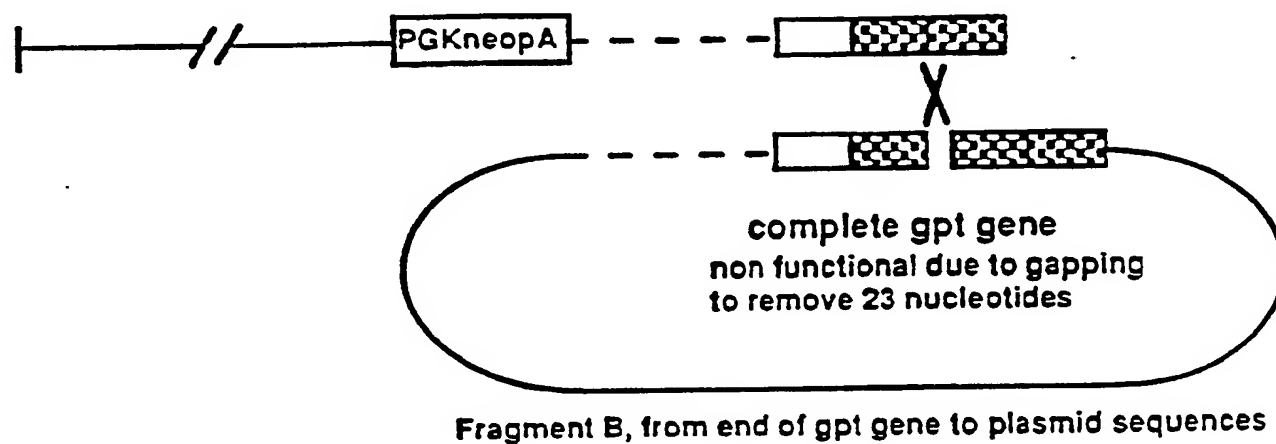
Step#2, homologous recombination: Adding the contiguous clone: Selection in HAT

FIGURE 6

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6C

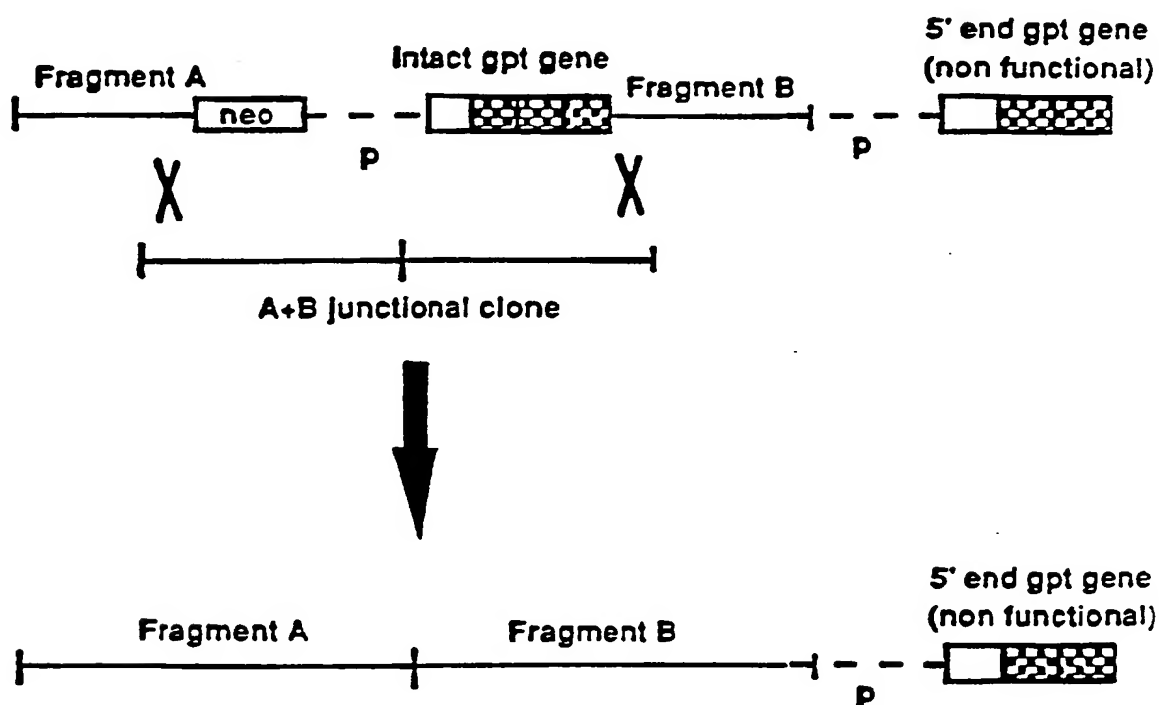
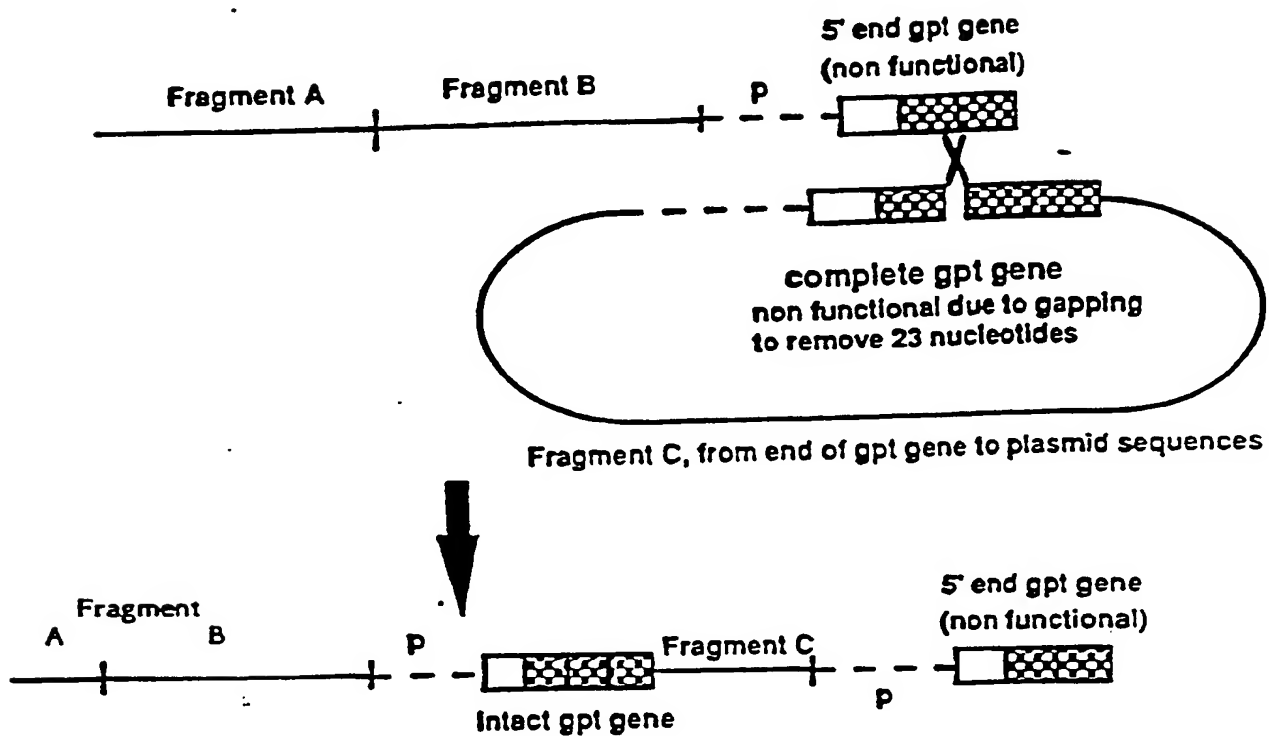
Step#3: Repair A-B junction. Select in - 6TG (100%)

FIGURE 6 (continued)

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6D

Step #4: Addition of contiguous clone

6E

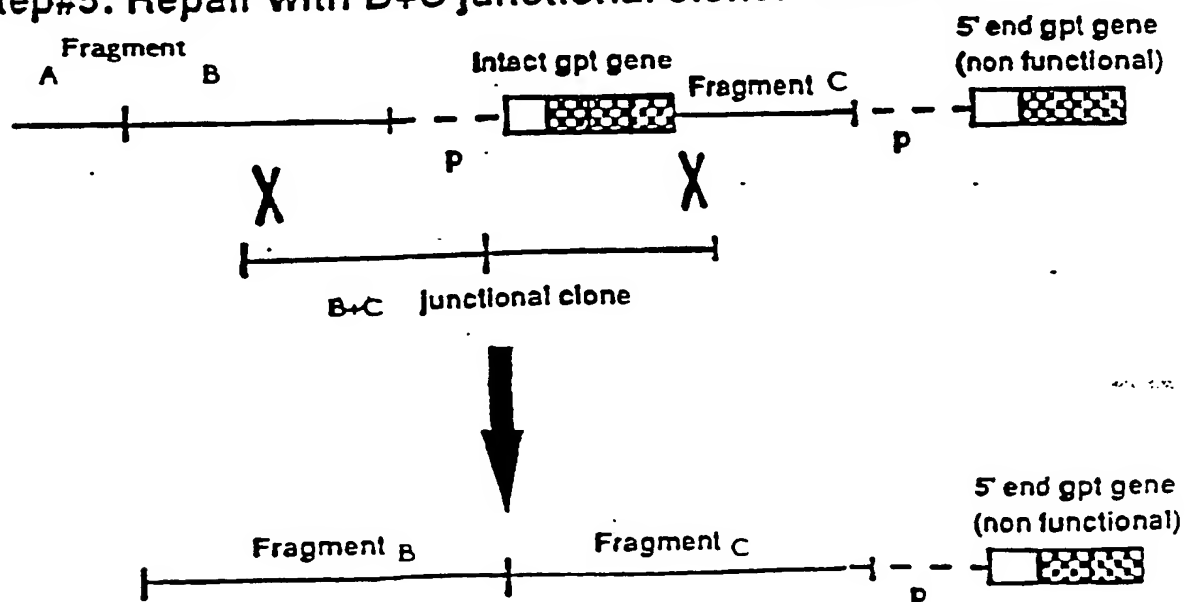
Step#5: Repair with B+C junctional clone. Select in - 6TG (100%)

FIGURE 6 (continued)

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Co-Electroporation

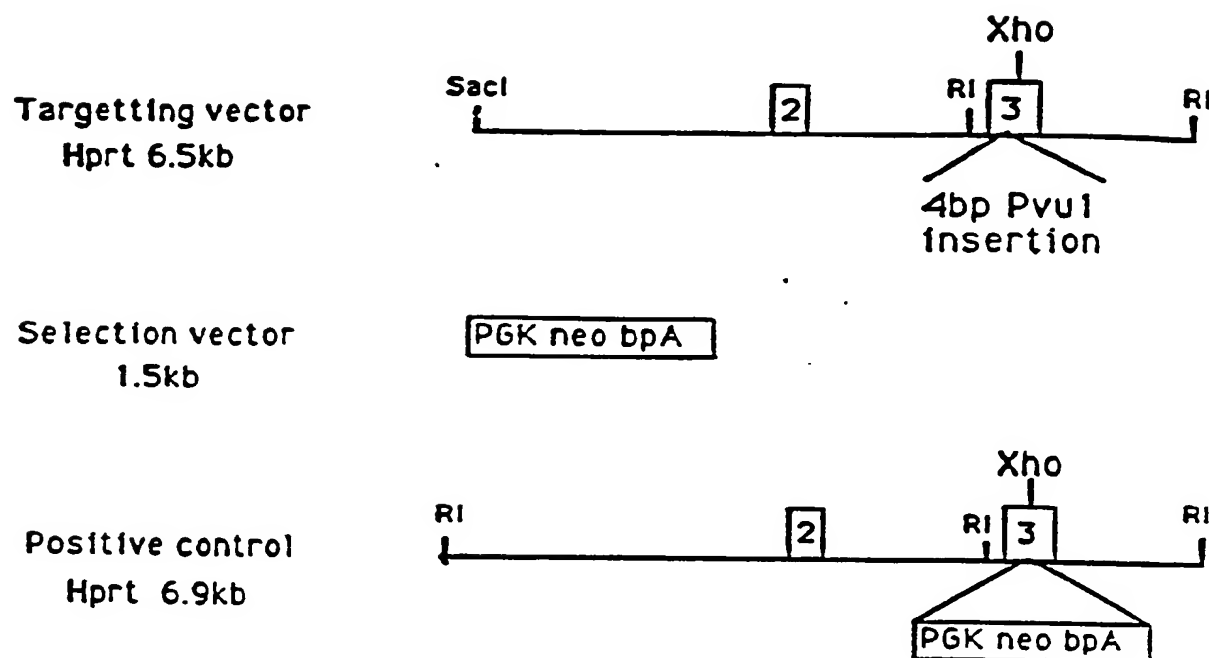


FIGURE 7

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Predicted structure of the *hprt* locus following homologous integration of the IV6.8 vector

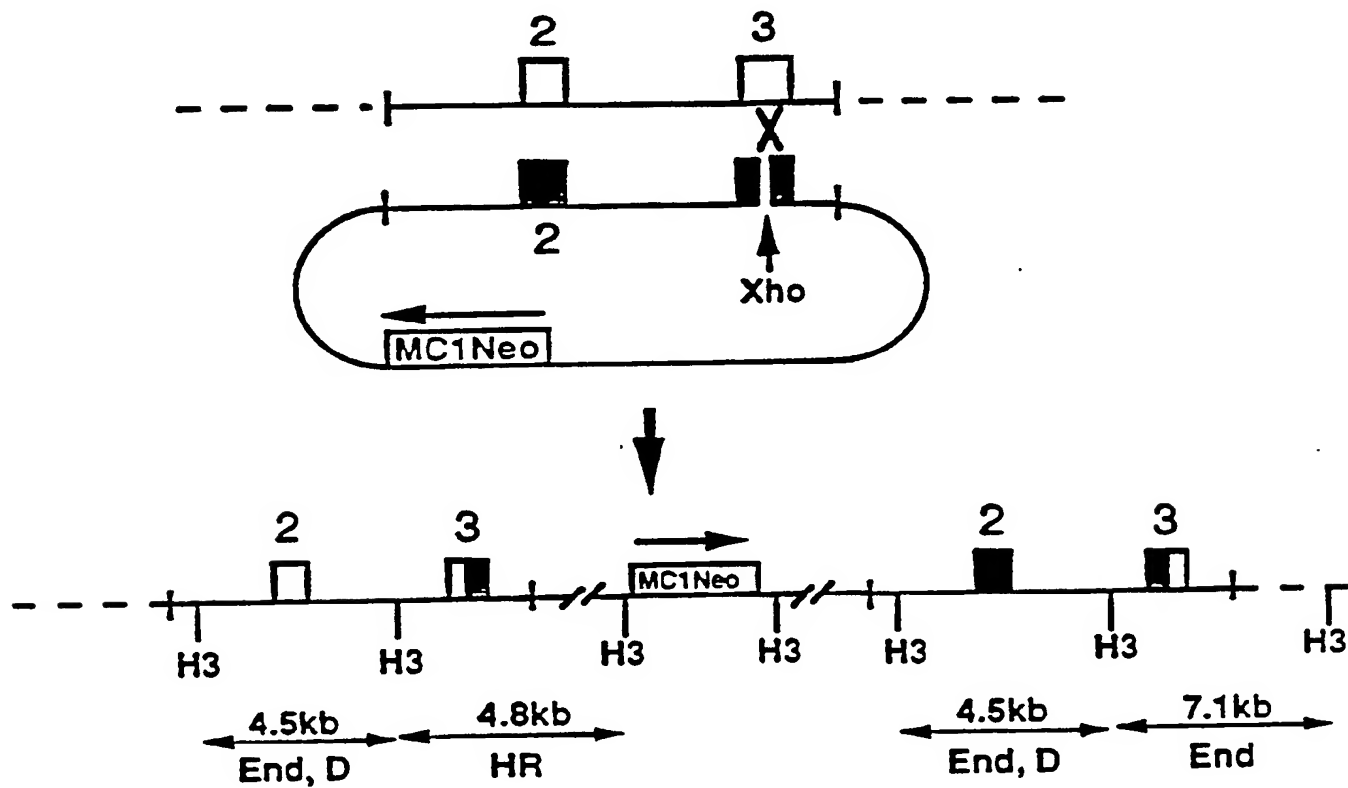


FIGURE 8

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Reversion of homologous recombinants generated with insertion vectors

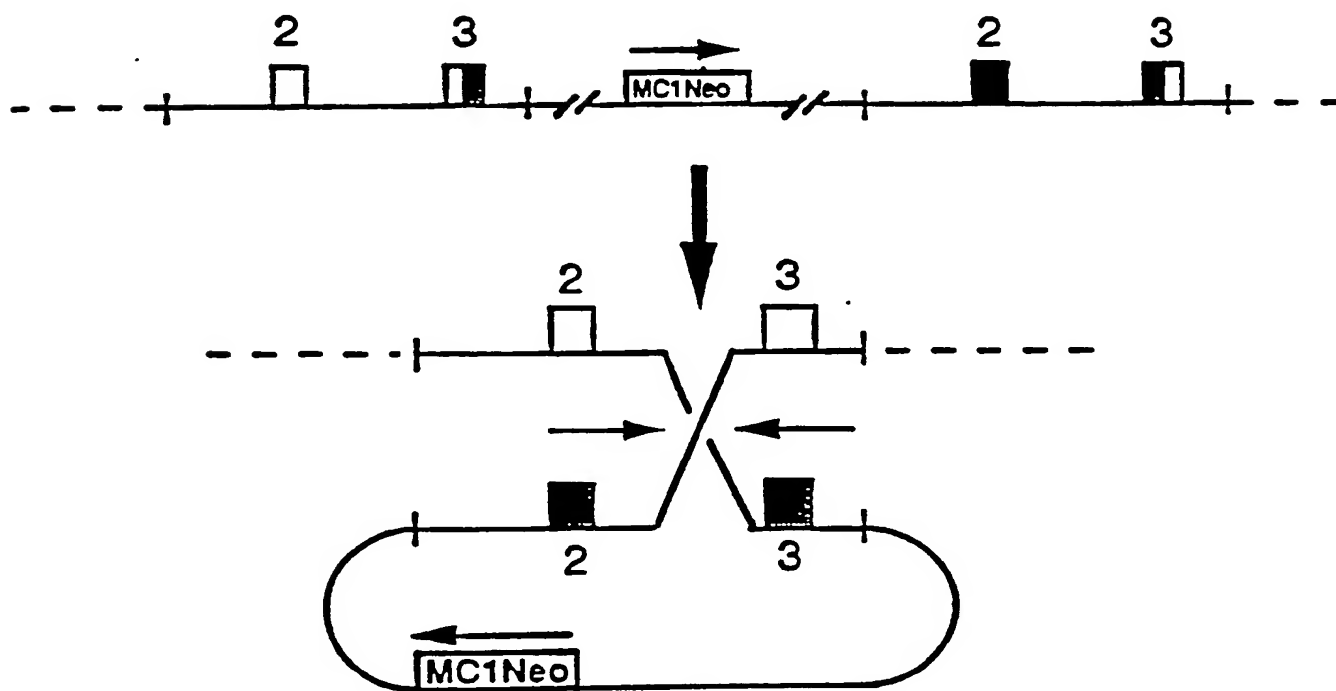


FIGURE 9

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Poly A selection

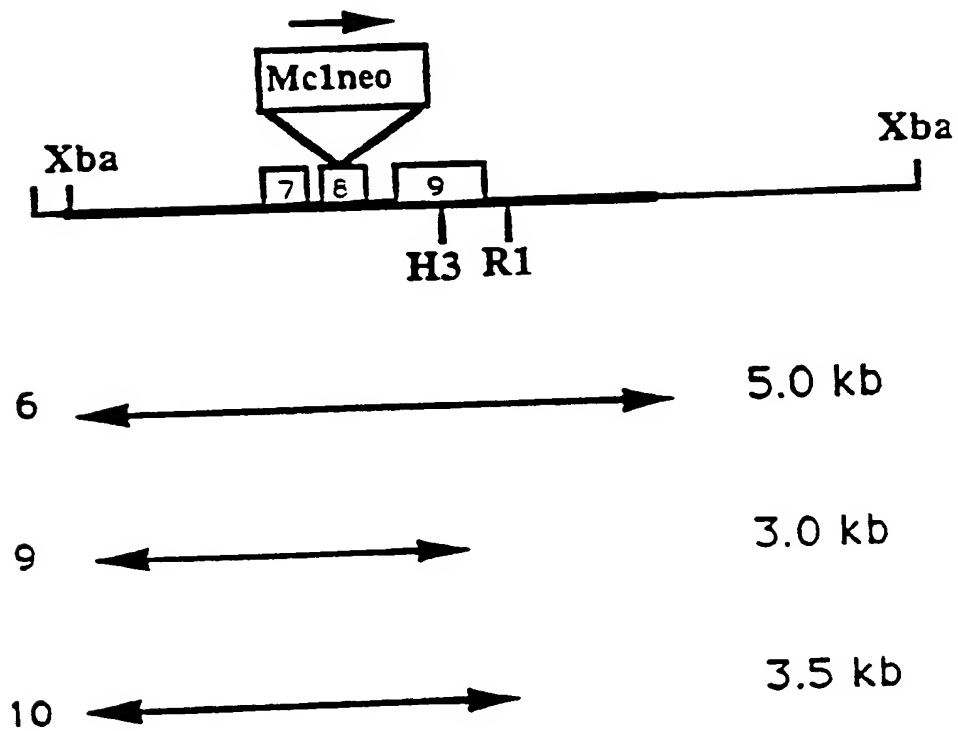


FIGURE 10

FIGURE 11

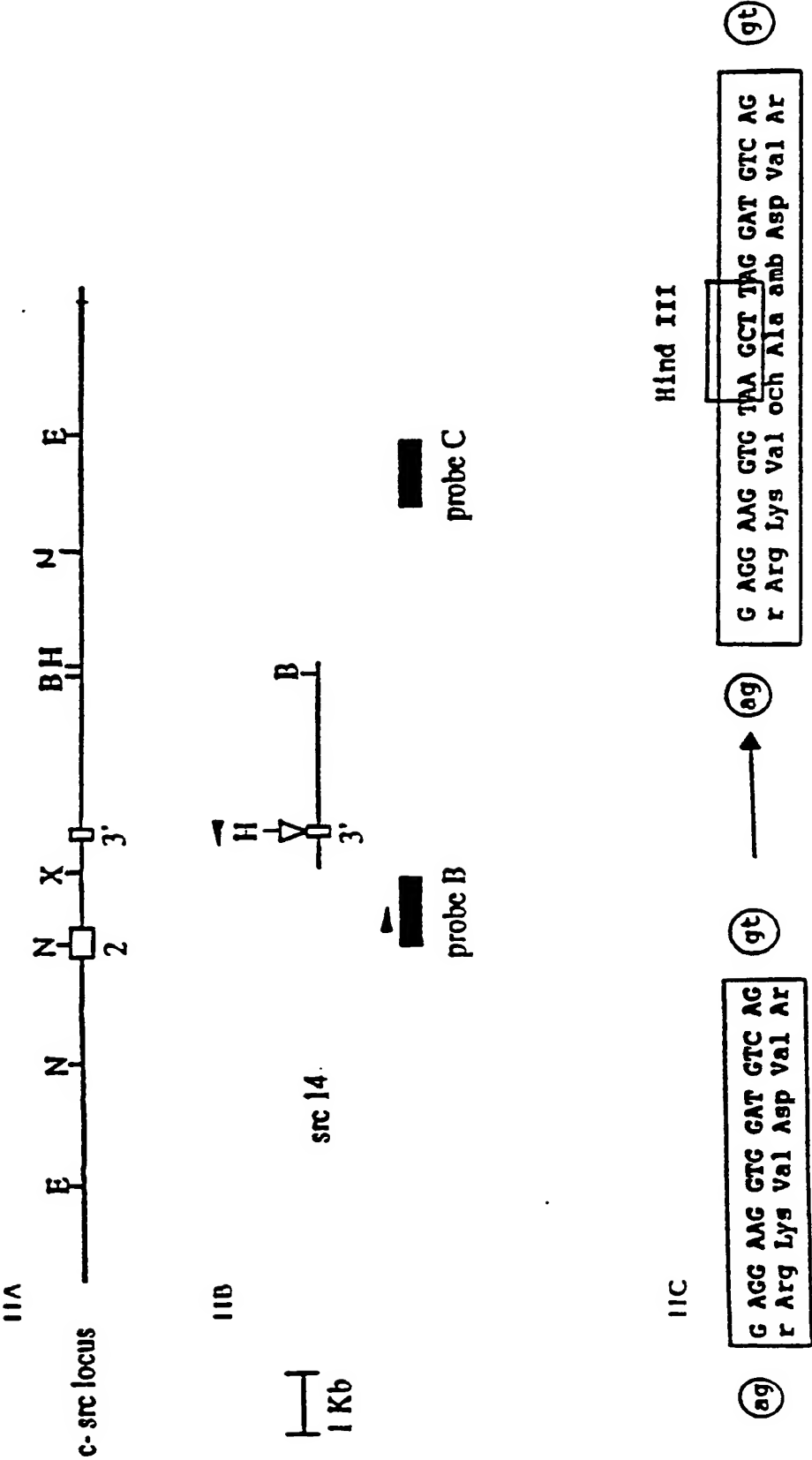
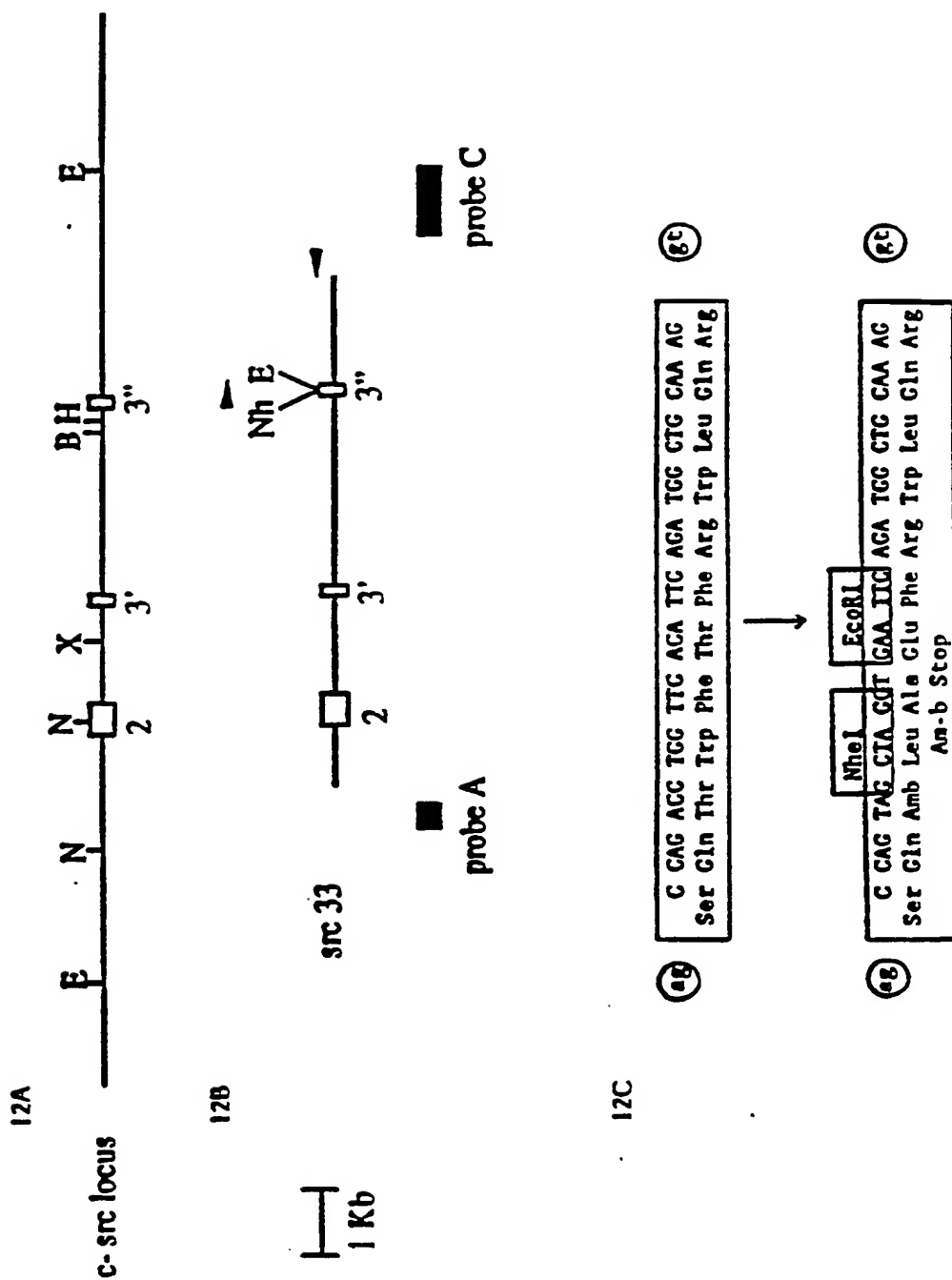


FIGURE 12



Direct selection for targeted recombination events

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Random Single copy or concatameric Integrations

Targeted

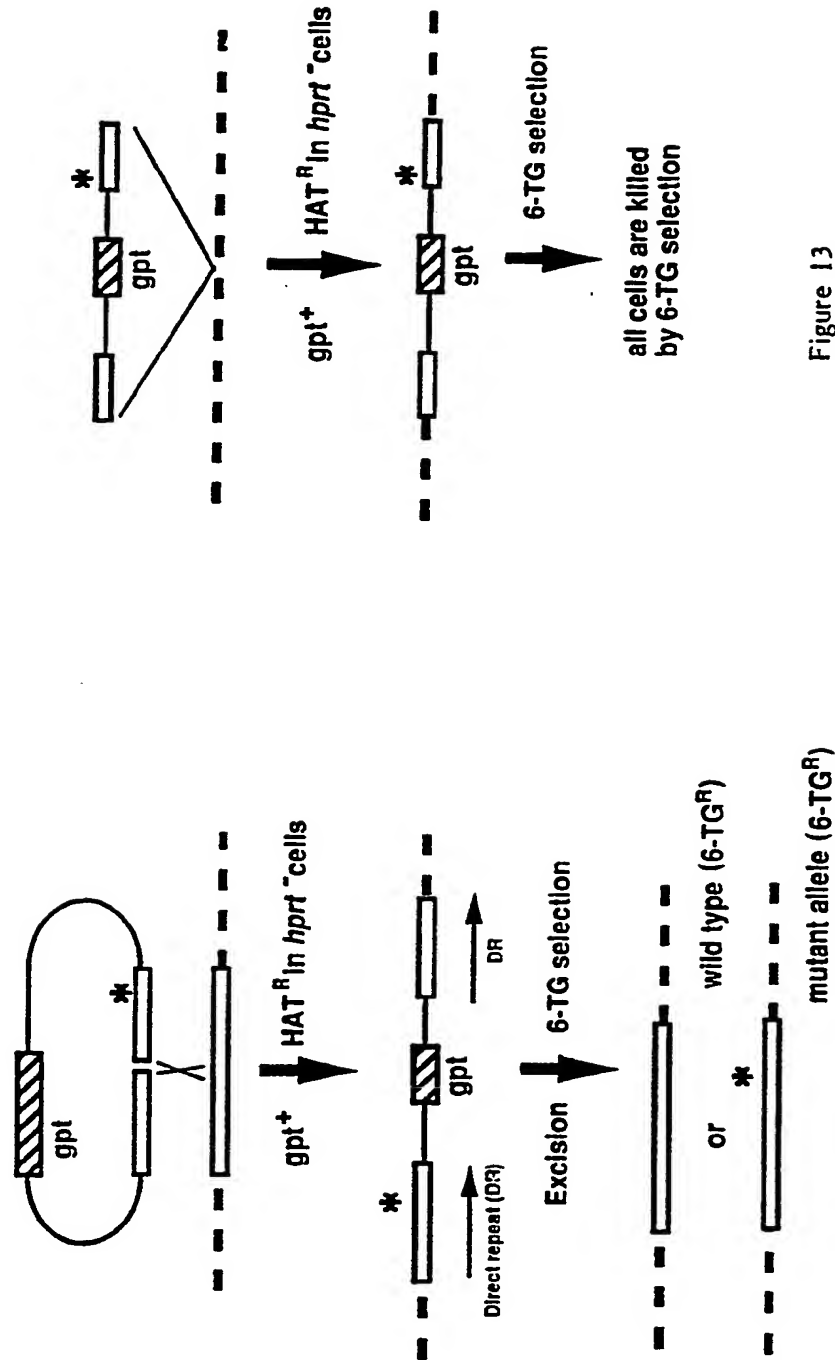


Figure 13

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US91/04006

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC I.P.C.(5): C12N 15/00, 5/00 U.S.Cl: 435/172.3, 240.1; 935/55, 56, 70														
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; padding: 5px;">U.S.</td> <td style="padding: 5px;">435/172.3, 240.1; 935/55, 56, 70</td> </tr> </table> <div style="text-align: center; padding: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U.S.	435/172.3, 240.1; 935/55, 56, 70								
Classification System	Classification Symbols													
U.S.	435/172.3, 240.1; 935/55, 56, 70													
APS, Chemical Abstracts, Biological Abstracts														
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%;">Category ¹⁰</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;"><u>X</u> <u>Y</u></td> <td style="padding: 5px;">Proceedings of the National Academy of Science, vol. 85, issued November 1988, Doetschman et al, "Targeted mutation of the Hrpt Gene in Mouse Embryonic Stem Cells," pages 8583-8587, see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-4, 6-16 <u>18-21, 34</u> 35-37</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;"><u>X</u> <u>Y</u></td> <td style="padding: 5px;">Science, Vol. 245, issued 15 September 1989, Johnson et al, "Targeting of Non expressed Genes in Embryonic Stem Cells via Homologous Recombination, pages 1234-1236, see entire document.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-4, 6-16 <u>18-21,</u> 34-37</td> </tr> <tr> <td colspan="3" style="text-align: center; padding: 20px;">(Next)</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	<u>X</u> <u>Y</u>	Proceedings of the National Academy of Science, vol. 85, issued November 1988, Doetschman et al, "Targeted mutation of the Hrpt Gene in Mouse Embryonic Stem Cells," pages 8583-8587, see entire document.	1-4, 6-16 <u>18-21, 34</u> 35-37	<u>X</u> <u>Y</u>	Science, Vol. 245, issued 15 September 1989, Johnson et al, "Targeting of Non expressed Genes in Embryonic Stem Cells via Homologous Recombination, pages 1234-1236, see entire document.	1-4, 6-16 <u>18-21,</u> 34-37	(Next)		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
<u>X</u> <u>Y</u>	Proceedings of the National Academy of Science, vol. 85, issued November 1988, Doetschman et al, "Targeted mutation of the Hrpt Gene in Mouse Embryonic Stem Cells," pages 8583-8587, see entire document.	1-4, 6-16 <u>18-21, 34</u> 35-37												
<u>X</u> <u>Y</u>	Science, Vol. 245, issued 15 September 1989, Johnson et al, "Targeting of Non expressed Genes in Embryonic Stem Cells via Homologous Recombination, pages 1234-1236, see entire document.	1-4, 6-16 <u>18-21,</u> 34-37												
(Next)														
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center;">28 August 1991</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">24 SEP 1991</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority <div style="text-align: center;">ISA/US</div> </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Official <div style="text-align: center;"> Deborah Couch </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center;">28 August 1991</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">24 SEP 1991</div>	International Searching Authority <div style="text-align: center;">ISA/US</div>	Signature of Authorized Official <div style="text-align: center;"> Deborah Couch </div>								
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International Searching Authority <div style="text-align: center;">ISA/US</div>	Signature of Authorized Official <div style="text-align: center;"> Deborah Couch </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
<p> <u>III</u> <u>V</u> </p>	<p> Molecular and Cellular Biology, vol. 10, issued February 1990, Baur et al, "Intermolecular Recombination in Plants," 492-500, see entire document. </p>	<p> 1-5, 12, 17 <u>19, 21.</u> 34-37 </p>
<p> <u>III</u> <u>V</u> </p>	<p> The Plant Cell, Vol. 2, issued May 1990, "Homologous Recombination in Plant Cells after <u>Agrobacterium</u> Mediated Transformation," pages 415-425, see entire document. </p>	<p> 1-5, 12, 17 <u>19, 21.</u> 34-37 </p>

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X
Y

Nature, Volume 338, issued 09 March 1989, Joyner et al, "Production of a mutation in Mouse Fu-2 Gene by Homologous Recombination in Embryonic Stem Cells," pages 153-156, see entire document.

1-4,6-16
18-21,
34-37

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. ☐ Claim numbers because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-21 and 34-37 telephone practice

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

